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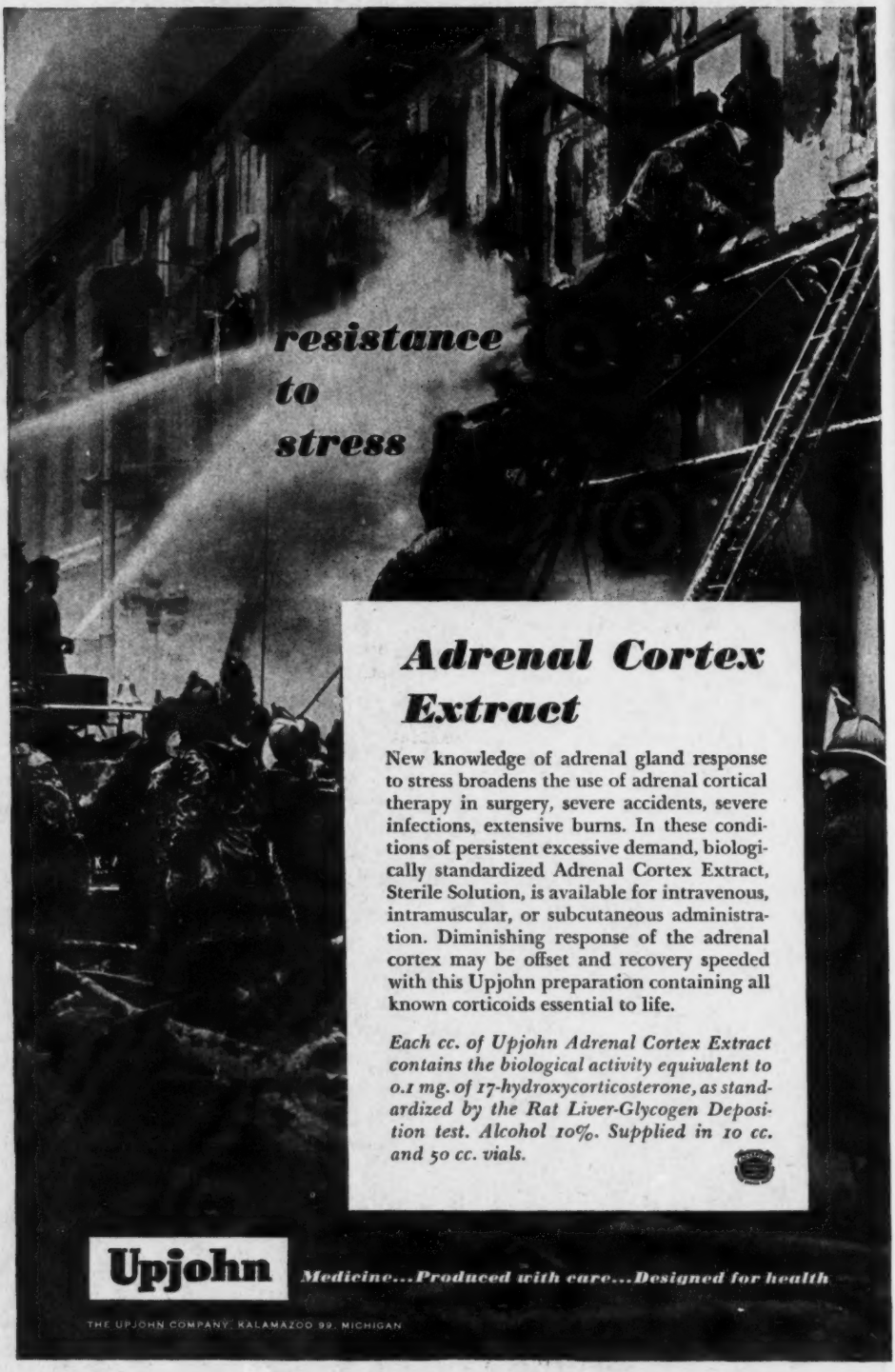

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ENDOCRINOLOGY

VOLUME 49 JULY, 1951 NUMBER 1

THE EFFECTS OF INJECTION OF LITHOSPERMUM RUDERALE ON THE ENDOCRINE ORGANS OF THE RAT

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THE earliest reference to the pharmacological action of the plant lithospermum ruderale was contained in a treatise on the medicinal uses of plants by the Indian tribes of Nevada (Train, Hendricks, and Archer, 1941). The plant was used by small groups of Indians as a means of contraception. If taken over a prolonged period of time the reputed result was permanent sterility.

In 1945, Cranston studied the action of the plant on mice. She found that a 50% alcoholic extract of lithosperm incorporated in the diet at a level of 40% caused cessation of the estrous cycle of mice in the diestrous phase. Even after several months of treatment, normal cycles resumed within six days following withdrawal of the plant. Powdered crude plant as 15% of the diet also caused cessation of the cycles without loss of body weight. The C_3H strain has been reported as demonstrating a more uniform and complete anestrous than Rockland mice (Drasher and Zahl, 1946). Lithosperm more recently has been found to inhibit the estrous cycle of rats, and to be at least ten times more active by subcutaneous injection than by oral feeding (Plunkett, Colpitts and Noble, 1950; Noble and Plunkett, 1950).

The prolonged administration of lithosperm at therapeutic levels caused an initial inhibition of growth, but by the hundredth day of treatment normal weight had been attained (Cranston, 1945). In ten day experiments, twenty-one day old mice on 40% lithosperm diets showed inhibition of weight increase in the pituitary, gonads, uterus and vagina, thymus, seminal vesicles and prostate (Cranston, 1945). After almost one year of treat-

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ment microscopic examination demonstrated a follicular atresia of the ovaries and mild uterine atrophy, while the thyroid and pituitary glands appeared normal (Zahl, 1948).

Cranston found that the actual food intake over and above the lithosperm fraction of the diet was greater in the treated animals than in controls. This, combined with a decreased weight gain during the greater part of the experiment, normal B.M.R. studies, and no anatomical changes in the thyroid glands presented a paradoxical picture for which no explanation was offered.

Mice which had been in prolonged diestrous, due to lithosperm treatment, developed cornified vaginal epithelium upon injection of 2 I.U. of estrone (Cranston, 1945). This was later confirmed in rats (Plunkett, Colpitts and Noble, 1950). Treated animals, though not responding to subcutaneous injection of 0.33 mgm. of a follicle stimulating gonadotrophin (Pituitary Synergist Parke Davis) in divided doses, did develop vaginal estrous with 3 mgm. of the same preparation (Cranston, 1945). The difference in weight response of the ovaries and uteri between the animals receiving gonadotrophin plus lithosperm, and gonadotrophin only, was not recorded.

Repeated experiments in this laboratory have shown that lithosperm when injected separately, markedly inhibits the trophic effect of various gonadotrophic preparations, e.g. pregnant mare's serum, chorionic gonadotrophin, sheep pituitary gonadotrophin and rat pituitaries (Noble, Taylor and Plunkett, 1950; Plunkett and Noble, 1950). Furthermore, lithosperm, in amounts as low as 0.8 mg. of lyophilized water extract of root, almost completely inhibits 100 I.U. of pregnant mare's serum, when mixed in vitro and incubated 2 hours at 37° C. prior to injection. This effect is most marked when hypophysectomized immature female rats are used (Plunkett and Noble, 1950).

The pituitary glands of lithosperm treated animals, when implanted in twenty-one day old female mice, caused less ovarian stimulation than those glands of normal controls (Cranston and Robinson, 1949). Drasher confirmed this work but reported that though the gonadotrophic content of the pituitaries of lithosperm treated mice was less than normal, it did increase normally when the animals were castrated, even though they were still receiving lithosperm (Drasher, 1949).

Further studies on the effect of lithosperm on the gonadotrophin content of the rat pituitary showed that after prolonged high dosage, the content, though somewhat decreased, was still high (Noble, Taylor and Plunkett, 1950). Lithosperm also contains a water soluble estrogen which causes uterine hypertrophy in immature rats when 20 mgm. of a lyophilized lithosperm tops extract are injected twice a day for five days. Also, when ap-

plied locally to the vaginas of castrated female rats, cornification of the vaginal epithelium may occur (Plunkett, Colpitts and Noble, 1950). This estrogen may account for the reported decreased gonadotrophin content of the pituitaries of treated animals.

Several European plants have been investigated by Zadina, and found to cause anestrous in the mouse. Polygonum hydropiper L., and Sanguisorba officinalis L. were the most effective and supressed cycles at a dose of 25% dry plant in the diet. Some activity was found in other species, but only when fed as 35–50% of the diet (Zadina, 1950).

Extensive experimental work has been conducted with lithosperm in this laboratory during the past three years. The effects on the sex cycle have been extended to rats, and the preparation of extracts of the plant for injection have led to a marked increase in potency. Experiments designed to determine the mode of action have been reviewed (Noble, Plunkett and Taylor, 1950). The present paper extends these studies and the effects of repeated daily doses of lithosperm have been noted on rats of different sex and age. Duplicate sets of experiments, run with the same extract at approximately a year's interval are shown since apparent deterioration of lithosperm extracts takes place.

METHODS

Sprague Dawley rats fed on Master's fox cubes were used for these experiments. The organs of the rats were weighed immediately on removal at autopsy, except in the case of the seminal vesicles and prostate which were previously fixed in formalin. Lyophilized aqueous extracts of the ground tops or roots of the lithospermum ruderale* were administered by subcutaneous injection as indicated. The total solid of such extracts was used to indicate the dose level employed. The aqueous extracts usually contained about one tenth the total solid of the dried plant starting material, in the case of the roots, and one third in the case of the tops.

RESULTS

Effect of lithosperm treatment on immature female rats

In October 1948, immature female rats of approximately 50 gms. in weight received daily injections of 20 mg. of lithosperm tops for thirty days, and were killed on the following day. The effect on the weights of the sex organs may be seen in Table 1.

This experiment was repeated in November 1949, with the same batch of lithosperm extract as previously used. Two groups of immature female rats about 50 gms. in weight were given daily injections of 20 mgm. of lithosperm tops for fifteen and twenty-two days respectively. The effect of this treatment on the weight of endocrine organs may be seen in Table 2.

* Lithosperm extracts were kindly supplied by Ayerst, McKenna and Harrison Ltd., Montreal, Canada.

TABLE 1. EFFECT OF LITHOSPERM TOP TREATMENT ON THE ENDOCRINE ORGAN WEIGHTS OF IMMATURE FEMALE RATS

	Untreated controls	20 mg. L.T./day×30	P value
No. of rats	10	13	
Body wt. (gm.)	147 ± 5*	119 ± 4	< .01
Ovaries (mg.)	45 ± 3	14 ± 1	< .01
Uteri (mg.)	263 ± 39	109 ± 24	< .01

* Standard error of the mean.
L.T. Lithosperm top.

It may be seen that the most striking action of extracts of lithosperm tops, particularly in the 1948 experiment, was the inhibition of the normal development of the ovaries, with a secondary reduction in size of the uterus. Apparently the effects of the extract may be powerful enough to prevent ovarian growth so that the organ remains in the infantile state. Some sex hormone production takes place since the uterus is increased in weight and though about seven days later than the controls, opening of the vagina does occur. Histologically the most prominent feature is the absence of mature follicles, ovulation and of corpora lutea in the treated

TABLE 2. EFFECT OF LITHOSPERM TOP TREATMENT ON THE ENDOCRINE ORGAN WEIGHTS OF IMMATURE FEMALE RATS. NOVEMBER 1949

	Untreated controls	20 mg. L.T./day×15	P value	Untreated controls	20 mg. L.T./day×22	P value
No. of rats	7	7	—	7	7	—
Body wt. (gm.)	129 ± 4*	108 ± 3	<0.01	137 ± 3	136 ± 5	>0.05
Ovaries (mg.)	33 ± 4	21 ± 4	>0.05	42 ± 2	31 ± 5	0.05
Uteri (mg.)	309 ±52	159 ±31	>0.05	281 ±34	152 ±20	<0.01
Adrenals (mg.)	33 ± 2	33 ± 2	—	37.9± 2.2	43 ±13	>0.05
Thymus (mg.)	418 ±26	317 ±23	<0.02	409 ±34	339 ±23	>0.05
Thyroid (mg.)	9.1± 0.5	7.6± 0.5	>0.05	9.8± 0.3	9.0± 0.3	>0.05
Pituitary (mg.)	4.4± 0.2	4.5± 0.3	>0.05	6.3± 0.2	5.9± 0.3	>0.05

* Standard error of the mean.
L.T. Lithosperm top.

animals. Sections taken every five hundred microns failed to show such development in any ovary of the 1948 series.

In the 1949 experiment the histological differences were less pronounced but the most consistent change was a diminished number of corpora lutea.

The prolonged injections of lithosperm were not without both local and general toxicity. The animals usually did not grow as well as did the controls. In the 1949 series there were slight changes in adrenal and thymus weights indicative of toxicity. The diminution in thyroid weight was associated with histological evidence of reduced secretory activity of the gland. Similarly more striking changes in the thyroid will be reported in other experiments.

Effect of lithosperm on immature male rats

In October 1948, two groups of immature male rats, of 50–60 gms. body weight, were given daily injections of 20 mg. of lithosperm tops, and 20 mg. of lithosperm roots respectively for thirty days, and autopsied on the following day. The weights of the sex organs may be seen in Table 3.

TABLE 3. EFFECT OF LITHOSPERM TREATMENT ON THE SEX ORGAN WEIGHTS OF IMMATURE MALE RATS. OCTOBER 1948

		Untreated controls	20 mg. L.T./day×30	20 mg. L.R./day×30	P value
No. of rats		11	13	6	—
Body wt.	(gm.)	207 ± 7*	160 ± 5	167 ± 8	<0.01
Testes	(mg.)	2500 ± 98	2230 ± 80	2349 ± 54	>0.05
Seminal vesicles	(mg.)	284 ± 18	121 ± 11	128 ± 17	<0.01
Prostate	(mg.)	506 ± 25	280 ± 23	313 ± 25	<0.01

* Standard error of the mean.
L.T. Lithosperm top.
L.R. Lithosperm root.

In December 1949, two groups of immature male rats, about 60 gm. in weight, were started on daily injections of 20 mg. of lithosperm tops. Since this was the same extract as that used in the earlier experiment, and since the 1949 results on the female rats indicated that the extract had decreased in potency, a change in dosage was made. From the 13th day of this experiment the dose was raised to 40 mgm. per day, in both groups. This continued to the 16th and 23rd days respectively, and the animals were killed on the following day. The weights of the endocrine organs may be seen in Table 4.

TABLE 4. EFFECT OF LITHOSPERM TREATMENT ON THE ENDOCRINE ORGAN WEIGHTS OF IMMATURE MALE RATS. DECEMBER 1949

		Untreated controls	20 mg. L.T./day×12 + 40 mg./day×4	P value	Untreated controls	20 mg. L.T./day×12 + 40 mg./day×11	P value
No. of rats		7	7	—	7	7	—
Body wt.	(gm.)	143 ± 5*	116 ± 3	<0.01	166 ± 8	141 ± 3	>0.02
Testes	(mg.)	1516 ± 153	886 ± 97	0.01	2019 ± 173	1175 ± 133	<0.01
Seminal vesicles	(mg.)	26 ± 6	11 ± 1	<0.05	67 ± 15	19 ± 3	<0.01
Prostate	(mg.)	150 ± 16	90 ± 6	<0.01	251 ± 21	103 ± 13	<0.01
Adrenal	(mg.)	29 ± 1	32 ± 0	<0.02	32 ± 1	35 ± 2	>0.05
Thymus	(mg.)	439 ± 23	359 ± 51	>0.05	519 ± 43	298 ± 38	<0.01
Thyroid	(mg.)	8.8 ± 0.4	7.4 ± 0.1	<0.01	11.1 ± 0.7	9.9 ± 0.4	>0.05
Pituitary	(mg.)	4.5 ± 0.2	4.1 ± 0.1	>0.05	4.8 ± 0.2	4.6 ± 0.02	>0.05

* Standard error of the mean.
L.T. Lithosperm top.

These results indicate that extracts of either lithosperm tops or roots may cause an interference with the development of normal gonad function in immature male rats. Although some reduction in weight of the testes was noted the most marked change was in the prostate and the seminal vesicles. Histological examination of the testes showed only slight changes.

The seminiferous tubules of the treated animals were well developed although there was some evidence of impaired spermatogenesis. The interstitial cells appeared well developed and as numerous as in the control animals despite their apparently reduced secretory capacity. As in the female animals an increase in adrenal weights and a decrease in the thymus and thyroid glands was noted.

Effect of lithosperm on adult male rats.

The endocrine organs of adult male rats injected daily for 14 days with 80 mg. of lithosperm root extract, and autopsied on the 15th day, showed effects similar to those of immature animals—Table 5.

TABLE 5. EFFECT OF LITHOSPERM TREATMENT ON THE SEX ORGANS AND THYROIDS OF ADULT MALE RATS

		Untreated controls	80 mg. L.R./day×14	P value
No. of rats		5	8	—
Body weight	(gm.)	287 ± 4*	212 ± 3	<0.01
Testes	(mg.)	3599 ± 59	3158 ± 93	<0.01
Seminal vesicles	(mg.)	962 ± 59	267 ± 42	<0.01
Prostate	(mg.)	1316 ± 43	416 ± 45	<0.01
Thyroid	(mg.)	13 ± 0.6	9 ± 0.4	<0.01

* Standard error of the mean.
L.R. Lithosperm root.

The testes showed moderate weight loss, while the seminal vesicles and prostates are markedly reduced in weight. The thyroids again showed a weight loss in the treated group and microscopic study revealed a low epithelium and increased colloid storage, when compared with controls.

DISCUSSION

The experiments reported in this paper indicate that prolonged treatment with lithosperm is capable of preventing normal gonadal development in immature rats. Both follicle and corpus luteum development were retarded in the ovaries and normal uterine growth was prevented. The maturation of the spermatogenic tissue in males was somewhat inhibited, and although the histological changes in the interstitial cells were less apparent, the lack of growth of the seminal vesicles and prostate in the treated group indicates an inadequate androgen production.

The increase in adrenal size and partial atrophy of the thymus together with a reduction in growth rate indicate that the relatively crude extracts used have toxic properties. Local tissue reaction was also noted at the site of the subcutaneous injections. The effects described in the endocrine organs, however, are not believed due to simple toxicity of the extracts. A more detailed study of the histological changes will be described when more highly purified extracts of lithosperm are available.

The reduced size of the thyroid glands of the treated animals is consistent with other observations which indicate that lithosperm inhibits the action of thyrotrophin as well as gonadotrophins.

Cranston reported that the activity of the dried plant decreased after storing, and it would appear from the above results that lyophilized water extracts also slowly deteriorate.

SUMMARY

The effect of the subcutaneous administration of lyophilized water extracts of lithospermum ruderale on the endocrine organs of the rat has been studied.

In both male and female immature rats, the extracts caused impaired development of the gonads and secondary sex organs. An inhibitory effect was also noted on the thyroid glands. Lithosperm caused an atrophy of the testes, seminal vesicles, prostate and thyroid of adult male rats.

The activity of the lyophilized extracts was observed to decrease with time.

ACKNOWLEDGMENTS

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LIPID DISTRIBUTION IN THE SERTOLI CELL AND LEYDIG CELL OF THE RAT TESTIS AS RELATED TO EXPERIMENTAL ALTERATIONS OF THE PITUITARY-GONAD SYSTEM[1]

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INTRODUCTION

NUMEROUS histochemical reactions have been suggested to determine the specific nature of the lipid contained within the Leydig cell primarily to find support for the hypothesis that Leydig cells produce androgen (Pollock, 1942; Dempsey and Wislocki, 1946; Ashbel and Seligman, 1949; Cain, 1950). Sertoli cell lipid has been investigated less extensively although histochemical studies indicate that it consists in part of cholesterol and cholesterol esters (Perlman, 1950).

It shall be the purpose of this paper to present our findings of the distribution of testicular lipids as they relate to certain alterations of the pituitary-gonad system with no attempt to determine the specific chemical nature of the lipid. In a previous communication we have related changes in distribution of testicular lipids to age (Lynch and Scott, 1950).

MATERIALS AND METHODS

Young mature 200 gm. albino rats of the Wistar and Sprague-Dawley strains were used for all experiments. No difference was noted in the normal picture or experimental response of the two strains; nevertheless, litter-mates of the same strain were used for each experiment. All hypophysectomized animals were furnished by The Hormone Assay Laboratories, Chicago. The sella turcica of these animals was examined at the time of autopsy to establish absence of the pituitary.

All animals were fed Purina Laboratory Chow ad libitum except the groups subjected to inanition which received 5 gm. daily, and the hypophysectomized animals which received in addition to the stock diet, supplementary stock canned dog-food which they apparently found more palatable and on which they better maintained body weight.

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All hormones[3] were injected subcutaneously, in sesame oil or in saline solution as indicated. In the case of the hypophysectomized animals, injections were initiated within 72 hours of operation. Most of the experiments were carried out over an arbitrary period of two weeks for the sake of consistency and comparability of results. In the case of the inanition experiments, the partial starvation was continued until the animals had lost the desired amount of weight, a period of about four weeks.

The experiments were controlled in the different studies by one of two methods. In some, one testis served as the control, while in others, the testes of litter-mates were used as controls. It was established that unilateral orchiectomy has no effect on the weight or microscopic appearance of the remaining testis.

All animals were sacrificed by ether anesthesia and the wet weights of the testes recorded. The testes were then fixed in 10% formalin for a period of 24 hours. Adjacent thin blocks of tissue were then used for hematoxylin and eosin and Oil Red O stained sections (Lillie, 1948). The blocks for the former were put through the usual paraffin routine. The latter blocks were washed, infiltrated progressively for a period of 24 hours in 2%, 4%, and 10% gelatin at 37° C. (Zwemer, 1923), and imbedded in 10% gelatin allowed to harden in the refrigerator for a period of one hour or longer. The gelatin blocks were trimmed and fixed in 10% formalin for 24 hours. Tissue sections were then cut at 40 microns on the frozen section microtome and floated in water. It was found that thinner sections would disintegrate, and that sections could not be cut at all without gelatin imbedding. After the sections were washed, they were stained with Oil Red O and mounted on glass slides with Kaiser's Glycerogel (Lillie, 1948).

Oil Red O, xylene-azo-xylene-azo-beta-naphthol, introduced by French (1926) and considered to be one of the best fat stains available in the Sudan group by Lillie, gives a deep scarlet color to lipids. Lison regards this group of "colorants" as defining the class of "lipoids" for the histochemist.

EXPERIMENTAL DESIGN AND RESULTS

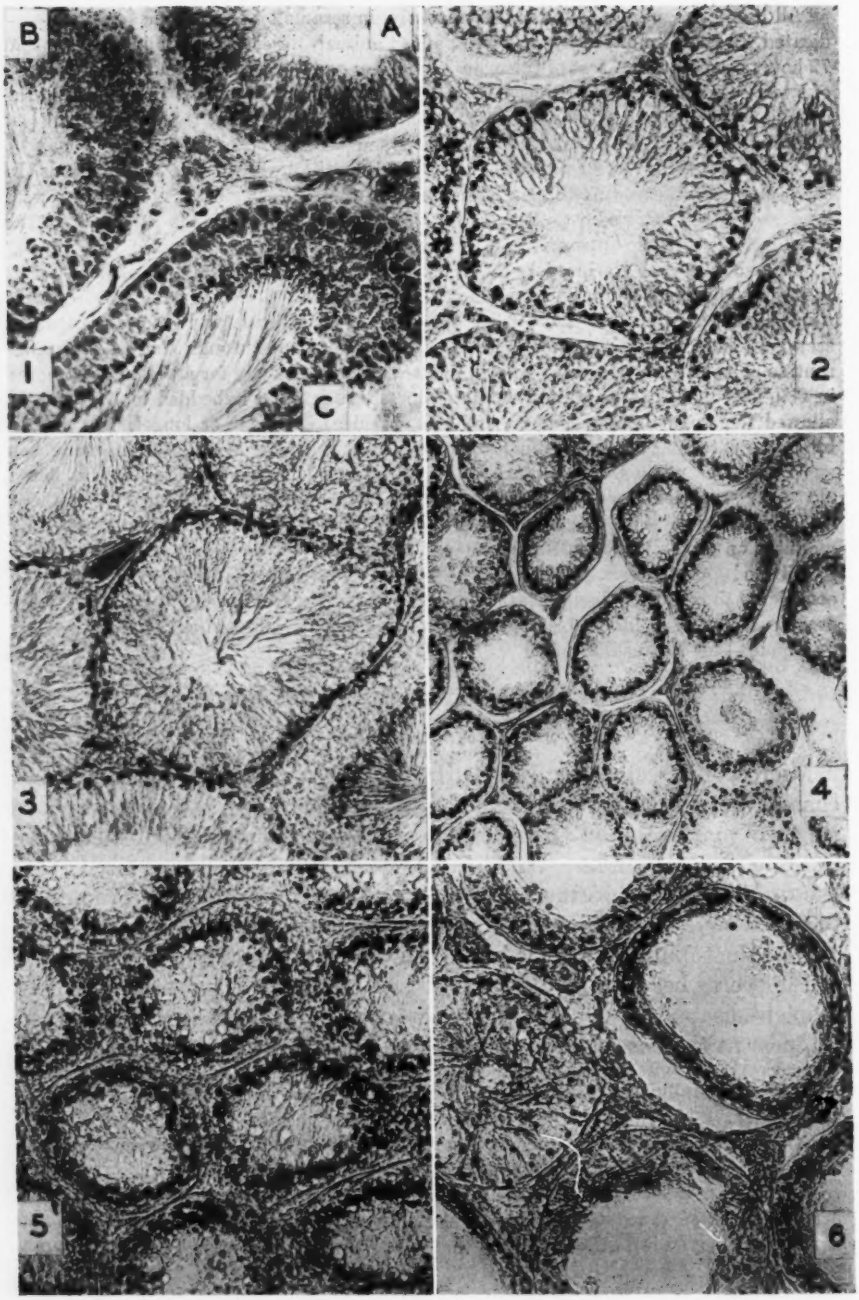
Normal testis

Microscopic sections stained with Oil Red O showed a consistent and characteristic lipid pattern in all control testes studied. There are various stages of maturation of sperm in the seminiferous tubules. Tubules in the "resting phase" with no spermatozoa present contain moderate-sized droplets of lipid at the basement membrane (Fig. 1A). As the sperm mature, these droplets seem to become smaller and more numerous and migrate toward the tubular lumen (Fig. 2B), finally occupying a position adjacent to the sperm heads (Fig. 1C) where they form a halo or rosette. These droplets disappear when the sperm are released into the lumen and then reappear at the basement membrane as a cycle continues. No lipid is seen

[3] TP in crystalline form was provided by Dr. Edward Henderson of The Schering Corporation.

Estradiol benzoate was provided by Dr. Edward Henderson of The Schering Corporation dissolved in sesame oil and labeled Progynon-B.

A.P. was provided by Dr. W. E. Hambourger of G. D. Searle and Company in the form of desiccated gonadotropic factor of sheep anterior pituitary gland labeled Gonadophysin and assayed in R.U.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in the Leydig cells of the normal albino rat, although it was well demonstrated in several wild rats studied.[4] These lipid droplets may be contained within Sertoli cells.

Effect of androgen in the intact rat

Four animals were injected subcutaneously every other day with 0.3 mg. TP in sesame oil for a period of 14 days. The only gross variation from the control group was in the weight of the testes, the treated animals averaging 0. 2 gm. less than the controls. Microscopically, there were more tubules in the "resting phase" than in the control testes and the lipid droplets at the basement membrane were larger than in the normal tubule. There was a marked accumulation of lipid in an occasional Leydig cell nest and the Leydig cells showed atrophy of the cytoplasm and pyknosis of the nuclei with the H and E stain.

Effect of estrogen in the intact rat

A pilot experiment demonstrated loss of testicular weight, diminished to absent spermatogenesis and an increase in the lipid in the Sertoli cell zone at the basement membrane. The Leydig cells contained no lipid and appeared normal with the H and E stain. Four groups (5 each) of animals receiving doses of estradiol benzoate varying from 0.01 mg. to 0.6 mg. in sesame oil every other day for approximately four weeks showed an overlapping of the response in the four groups. All groups failed to gain body weight at the same rate as untreated animals. There was a failure to gain weight or a loss of testis weight in all of the treated animals and the micro-

[4] Provided by Dr. Curt Richter, Phipps Institute, Johns Hopkins Hospital.

PLATE 1

All the sections illustrated in this plate were stained with Oil Red O

FIG. 1. Testis of 200 gm. normal albino rat. ×150. A. Tubule in "resting" phase with lipid droplets at basement membrane. B. Tubule in "intermediate" phase with droplets "migrating." C. Tubule in mature phase with droplets adjacent to the heads of the spermatozoa.

FIG. 2. Testis of rat subjected to inanition showing increase in number of tubules in the "resting" phase with an increase in the size of the lipid droplets at the basement membrane. ×150.

FIG. 3. Testis of rat showing minimal effect of injected estradiol benzoate. There is an increase in size of the lipid droplets in the "resting" phase. ×150.

FIG. 4. Testis of rat showing marked effect of injected estradiol benzoate. ×80. There is degeneration of the germinal epithelium and large drops of lipid in the Sertoli cells.

FIG. 5. Testis of rat 17 days after hypophysectomy showing degeneration of germinal epithelium and large drops of lipid in the Sertoli cells. ×80.

FIG. 6. Testis of rat after 2 weeks of artificial cryptorchidism showing degeneration of germinal epithelium, increase in Sertoli cell lipid and Leydig cell hyperplasia. ×150.

scopic changes described above were present in varying degrees in all of the testes (Figs. 3 and 4).

Effect of hypophysectomy

Seven animals were sacrificed 17 days following surgical extirpation of the pituitary gland. They failed to gain weight at the same rate as control animals and showed marked loss of testis weight (this was substantiated subsequently in a group of 41 animals utilized for purposes of testis extraction). Microscopically (Fig. 5), there was a diminution in the size of the seminiferous tubules, almost complete degeneration of the germinal epithelium and atrophy of the Leydig cells with nuclear pyknosis. The Sertoli cells contained large droplets of lipid; no lipid was demonstrated in the Leydig cells.

Effect of artificial cryptorchidism

Three animals were rendered experimentally cryptorchid on one side by suturing the testis to the abdominal wall so as to prevent passage of the testis into the scrotal sac. They were sacrificed two weeks later at which time there was a marked loss of testis weight in the abdominal testis as compared to the scrotal testis. Microscopically (Fig. 6), there was a loss of germinal epithelium with an accumulation of lipid in the Sertoli cells. There was an hyperplasia of the Leydig cells which contained no lipid.

Effect of inanition in the intact rat

Four animals were subjected to slow starvation on a diet of 5 gm. Purina Laboratory Chow daily until they had lost approximately ½ their previous body weight. While there was no appreciable loss of testis weight, there was a failure to gain weight in the normal manner and microscopically there was atrophy of Leydig cells, germinal epithelium and an increase in Sertoli cell lipid (Fig. 2).

Effect of AP in the hypophysectomized rat (Gonadophysin)[3]

Four animals received 4 R.U. Gonadophysin every other day for 14 days. The testis weights were fairly well maintained and normal spermatogenesis was present in about 25% of the tubules on microscopic examination. The Leydig cells were normal in appearance in the H and E stained sections.

DISCUSSION

Histochemical study of the lipids in the testis of the young adult albino rat revealed a constant and characteristic picture. The "migration" of the Sertoli cell lipid from a position at the basement membrane to a position adjacent to the heads of the mature sperm is compatible with the belief

that the spermia are temporarily enclosed in the tips of the Sertoli cells for protection and nutrition (Maximow and Bloom, 1935). The absence of demonstrable lipid in the normal Leydig cells seems to be unique in the albino rat, as it was demonstrated in considerable quantities in the wild rat,[4] guinea pig, dog and human. This could be explained by rapid release of the elaborated lipid from the normally active albino rat Leydig cell. This is supported by the fact that lipid was demonstrated in the Leydig cells of animals treated with androgen which presumably inhibits the pituitary.

The authors believe that the increase in Sertoli cell lipid in the estrogen treated animals, the animals subjected to inanition and the hypophysectomized animals is due in the first to inhibition of the pituitary with resultant decreased FSH secretion, in the second to decreased FSH secretion secondary to pituitary atrophy and in the latter to the complete absence of FSH. That the atrophic effect of estrogens on the testis is due to their influence in arresting the output of gonadotropins from the pituitary was suggested by Moore (1930). This was substantiated by De Jongh (1940) who removed the pituitaries from rats and thereafter prevented atrophy of the testes by giving a combination of pituitary and placental gonadotropins. More recently, Howard, Sniffen, Simmons and Albright (1950) have described degeneration of the germinal epithelium and increase in Sertoli cell lipid in men with low FSH levels. Wislocki (1949) has shown diminished Leydig cell lipid and increased Sertoli cell lipid associated with absent spermatogenesis in the deer during the non-rutting season which may be due to lowered pituitary secretion of FSH and LH during this period.

It has previously been stated that "it is probably that the Leydig cell hormone inhibits LH secretion" (Howard, Sniffen, Simmons and Albright, 1950). This was supported in our experiments in that injections of TP resulted in atrophy of the Leydig cells indicating inhibition of LH.

That the Sertoli cell "stores" or is unable to liberate lipid in the absence of spermatogenesis is indicated by the accumulation of the lipid in the Sertoli cells of the experimentally cryptorchid testis where degeneration of the germinal epithelium results from the increased heat of the abdominal cavity (Moore, 1924).

The experiment concerned with the replacement of the extirpated pituitary by injected gonadotropins reaffirms the established fact that the testis can be maintained by administered A.P.

SUMMARY

The lipid "pattern" in the normal adult albino rat and the effect on this "pattern" of estrogen administration, androgen administration, inanition, artificial cryptorchidism and hypophysectomy is described.

The distribution of the tubular lipid, which appears to be in the Sertoli cells, in relation to the maturing sperm is compatible with the hypothesis that the Sertoli cells have a nutritive function. It is noted that in the absence of spermatogenesis, whatever the cause, the lipid accumulates in the Sertoli cell adjacent to the basement membrane of the tubule.

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A COMPARISON OF EXOPHTHALMOS AND TISSUE CHANGES INDUCED BY ACTIVE AND INACTIVATED THYROTROPHIC HORMONE*

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IT HAS been demonstrated by one of us (Dobyns 1946a, 1946b, 1946c) that several extracts of the anterior pituitary, rich in thyrotrophic hormone, when administered to guinea pigs produce exophthalmos and unusual tissue changes which can be demonstrated by special histologic techniques in the connective tissue, muscle, liver, kidney, lung, spleen, and thryoid. Fat in abnormal quantities was demonstrable in the liver, muscle, kidney and thyroid epithelium within twenty-four hours following the administration of a single large dose of the hormone preparation. Round cells similar to those described in the orbit and in certain muscles by Paulson (1937, 1939, 1940), Aird (1940, 1941), Brock (1941) and Smelser (1936, 1937, 1939) were found to contain lipoid droplets suggesting that the round cells were phagocytizing fat. The histologic evidence for the deposition of fat in the various tissues and the cellular infiltration accompanied by changes suggesting edema occurred concurrently with the development of the exophthalmos. These changes occurred in both intact and thyroidectomized animals and were produced with several preparations of pituitary thyrotrophic hormone. Because of the impurities in all of these preparations, one cannot say that the factors which produced exophthalmos, the histologic changes, and thyroid stimulation are one and the same hormone.

It has been demonstrated by Rawson, Sterne and Aub (1941, 1942) that with tissue culture technics the thyrotrophic hormone of a pituitary extract can be inactivated by exposure to explants of thyroid tissue. With

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* From the Mayo Foundation, Rochester, Minnesota and the Thyroid Clinic of the Massachusetts General Hospital, Boston, Massachusetts. Experimental work carried out in the laboratory of Dr. G. M. Higgins at the Institute of Experimental Medicine, Mayo Foundation by Dr. Dobyns, formerly Fellow in Surgery, Mayo Foundation; and by Dr. Rawson, formerly Henry P. Walcott Fellow, Harvard University, in the Thyroid Clinic of the Massachusetts General Hospital.

the same technics other tissues, excepting thymus and lymph nodes, were found to have no effect on the action of exposed thyrotrophic hormone. Rawson (1949) has also studied the effect of bathing thyroid slices in a medium containing a pituitary extract, rich in thyrotrophic and gonadotrophic hormone on the physiologic activity of both of these hormones. In this study it was observed that the gonadotrophic hormone is not altered by exposure to thyroid slices whereas the thyroid-stimulating effect is lost. Thus it was demonstrated that the inactivation of thyroid-stimulating hormone by thyroid tissue is not a non-specific one in which all pituitary hormones are altered by thyroid tissue. Indeed, it lends support to the thesis that the reaction is a specific one which may be of physiologic importance.

Since the thyroid-stimulating effect of thyrotrophic hormone is inactivated by incubation with explants of thyroid tissue, it was considered desirable to determine whether the exophthalmos-producing factor of a pituitary extract rich in thyrotrophic hormone was likewise inactivated by exposure to thyroid tissue. It was decided, therefore, to inactivate the thyrotrophic hormone of one of the more potent exophthalmos-producing extracts by exposure to explants of thyroid tissue and then to determine the capacity of this preparation to produce exophthalmos and the histologic changes referred to previously.

METHODS

Rabbit thyroids were explanted with tissue culture technics and bathed in 20 cc. of Tyrode's solution containing 0.5 Junkmann-Schoeller unit of thyrotrophic hormone* per cubic centimeter for twenty-four periods. The bathing fluids of many such incubations were pooled and assayed on chicks for thyrotrophic activity by the histometric method of Rawson and Salter (1940). By this method it was observed that the thyroid-stimulating activity had been lost. The inactivated hormone contained in the Tyrode's solution was then concentrated by acetone precipitation. A control preparation of thyrotrophic hormone in a similar concentration which had not been exposed to thyroid tissue was extracted in a similar manner from Tyrode's solution.

Nine male guinea pigs weighing between 330 and 400 gm. were chosen for the subsequent experiments. Efforts were made to avoid animals which gave any evidence of spontaneous exophthalmos. All the animals were thyroidectomized and the changes of the intercorneal distance were followed by multiple daily readings. Two improved methods of measuring minute changes in the prominence of the eyes, (intercorneal distance) described by one of us (Dobyns 1945) elsewhere, were used to measure exophthalmos in these animals. These measurements were made for thirteen days following thyroidectomy before the hormone preparations were given. Following this control period, three animals received the equivalent of 25 units** of inactivated thyrotrophic hormone daily for seven days. Three other animals received 25 units of the active thyro-

* A pituitary extract of the thyrotrophic hormone was kindly supplied by Parke Davis and Company.

** Potency based on the assay of the substance before inactivation.

trophic hormone daily for the same period. The remaining three animals served as thyroidectomized controls. The body weight and intercorneal distance (multiple determinations) were observed each day during the period of treatment.

In previous investigations (Dobyns 1946b. 1946c), it had been observed that striking changes in tissues were recognizable twenty-four hours after a single injection of 50 units of thyrotrophic hormone. Such changes that were found in fat were less striking after one week of treatment with thyrotrophic hormone than after twenty-four hours. For this reason, another acute experiment was planned to determine the tissue changes which might be induced by the inactivated thyrotrophic hormone as compared with those changes known to be produced by the active thyrotrophic hormone.

Another eight thyroidectomized animals were divided into two equal groups. The animals of one group received a single subcutaneous injection of 50 units** of inactivated thyrotrophic hormone. The animals of the other group received 50* units of the active

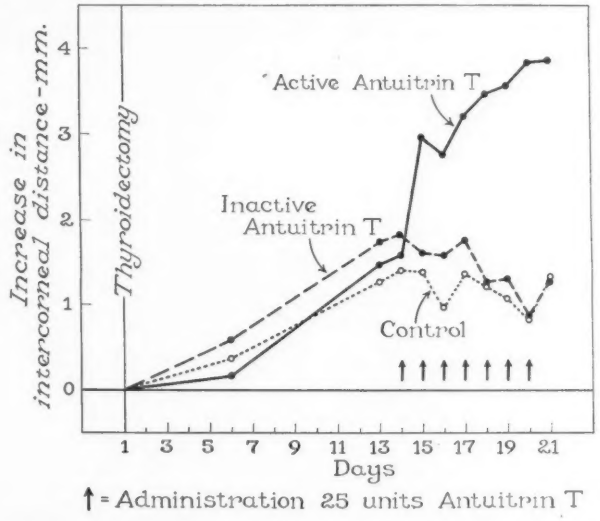

FIG. 1. Differences in effect of active and of inactivated thyrotrophic hormone on thyroidectomized guinea pigs. *a.* Animals that had received active thyrotrophic hormone. *b.* Thyroidectomized controls. *c.* Animals that had received inactivated thyrotrophic hormone.

thyrotrophic hormone. All animals were killed in twenty-four hours. Samples of tissue from the temporal (skeletal) muscle, cardiac muscle, lung, spleen, liver, kidney and several fat depots, including the cervical, axillary periureteral, orbital and testicular fat, were obtained. In addition, the entire orbital contents enclosed within the periorbital connective tissue sheath, the periorbita, were removed intact. These tissues were fixed in 10 per cent formalin, sectioned on a freezing microtome and stained with scarlet red and hematoxylin.

RESULTS

It was found that in those animals receiving the active thyrotrophic hormone, definite measurable exophthalmos developed within twenty-

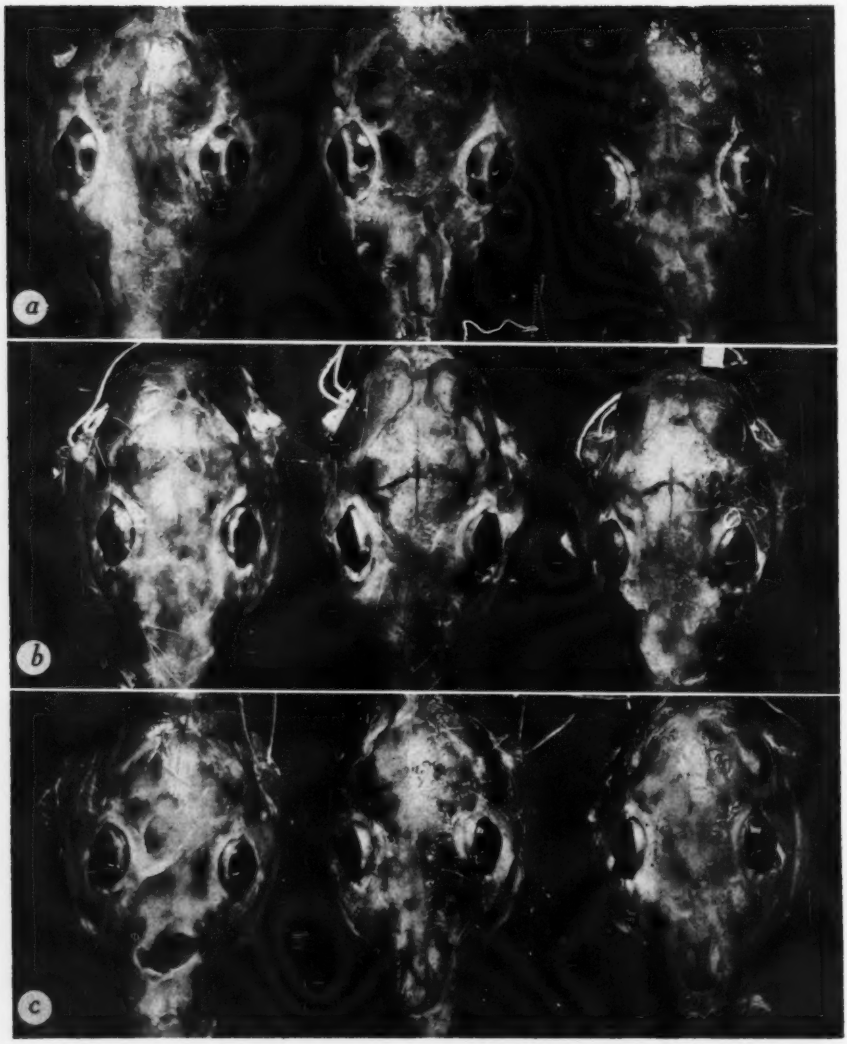

FIG. 2. Effect of active and of inactivated thyrotrophic hormone on exophthalmos.

four hours after the first adminstration of the hormone. After continued daily administration, the animals attained a degree of exophthalmos which was grossly obvious. In contrast, the inactivated thyrotrophic hormone did not produce exophthalmos even when observed with accurate methods of measurement.

The changes in the eyes of this second group of animals which received the inactivated thyrotrophic hormone followed the same course as in the eyes of animals which had been only thyroidectomized and observed as controls. Figure 1 represents the average of the changes of all three animals of each group. After the animals had been sacrificed, the heads were skinned and photographed (See Figure 2).

In the second experiment, it was observed that the inactivated hormone was incapable of producing the tissue changes induced by the active hormone preparation (Dobyns 1946b, 1946c). The active thyrotrophic hormone caused the appearance of large amounts of fat in the liver, in muscle fibers (including the cardiac, skeletal and extra-ocular), the kidney and the reticulo-endothelial cells. The active thyrotrophic hormone also caused the appearance of many polymorphonuclear leukocytes, tissue macrophages, and lymphocytes in connective tissue throughout the body. The two former types of cells were found to contain many fine droplets of fat. This tissue change was graded 3 or 4 on a basis of 1 to 4, in which 1 represents the least change and 4 the greatest (See Table 1).

TABLE 1. A COMPARISON OF CHANGES* INDUCED IN TISSUES BY ACTIVE AND INACTIVATED THYROTROPHIC HORMONE

	Phagocytosis in new cellular elements of		Increase in fat content of tissues			
	Orbital tissue	Perureteric fat	Extra-ocular muscles	Skeletal muscles	Liver cells	Kidney epithelium
Animals receiving inactivated thyrotrophic hormone	0	0	1	1	1	0
	0	0	1**	0	1	0
	0	0	2	1	2	0
	0	0	1	1	1	0
Animals receiving active thyrotrophic hormone	3	2	1**	1	3	2
	3	4	4	4	3	3
	2	4	1**	3	3	2
	4	3	4	4	4	3

* Graded on a basis of 1 to 4.
** Tissue not studied.

The inactivated thyrotrophic hormone caused little if any edema or increase of cellular elements in connective tissue and fat depots. It was also observed that the inactivated thyrotrophic hormone produced relatively little increase in the fat in the organs studied (See Table 1).

SUMMARY

A pituitary extract rich in thyrotrophic hormone loses its thyroid-stimulating quality after incubation with explants of thyroid tissue. It has been demonstrated that the exophthalmos-producing factor of this inactivated pituitary extract is also lost. Similarly the capacity to produce histologic changes, consisting of alterations in the fat and the cellular reaction in the connective tissue throughout the body associated with experimental exophthalmos are inactivated.

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EFFECT OF EXCESS IODIDE UPON ANTERIOR PITUITARY CYTOLOGY OF THE COMPLETELY THYROIDECTOMIZED RAT AND ITS BEARING ON THE QUESTION OF EXTRATHYROIDAL THYROXINE SYNTHESIS[1]

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ALTHOUGH cytological changes in the anterior pituitary after thyroidectomy were observed as early as 1889 (Rogowitsch), it has only recently been shown that the state of the acidophils in the rat anterior pituitary is related to the level of circulating thyroxine. Thus, Greisbach and Purves (1945) and Goldberg and Chaikoff (1950a) demonstrated that in the totally thyroidectomized rat, the acidophils constitute only about 1 per cent of the total adenohypophyseal cells, in contrast to a normal value of approximately 40 per cent. The daily administration of 0.5 gamma of DL-thyroxine per 100 gm. body weight does not prevent the appearance of typical thyroxine deficiency changes in the basophils of the thyroidectomized rat, but does keep the acidophils *normal* as judged by their morphological appearance and their percentage. The sensitivity of the acidophil response to thyroxine is most clearly brought out by comparing the effects of smaller doses; the daily injection of 0.25 gamma DL-thyroxine per 100 gm. body weight will maintain the acidophils at about half the normal value, whereas a dosage of 0.1 gamma results in just perceptible retention of granulated acidophils.

The sensitive response of the anterior pituitary has already been used to establish completeness of thyroidectomy (Goldberg and Chaikoff, 1949; 1950a; Griesbach and Purves, 1943) and to assess hormonal activity of thyroxine analogues (Greisbach *et al.*, 1949; Kennedy and Griesbach, 1949). In the present study we have applied it to the problem of thyroxine formation in sites other than the thyroid gland.

EXPERIMENTAL

Male and female rats of the Long-Evans strain, that had been raised on a stock diet (Taurog and Chaikoff, 1946) and that weighed from 90 to 110 gms., were used. Their

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[2] Atomic Energy Commission Fellow.

thyroids were completely destroyed by two injections of carrier-free I^{131} (500 μc. and 400 μc.) separated by an interval of one month. One month after the second injection, the rats were divided into three groups and treated as follows: The rats of one group (B) were fed the stock diet, and these served as radiothyroidectomized controls. The rats of group C were injected with 1000 gamma of iodide as KI every 8 hours for 28 days; during this interval they were fed the regular stock diet. The rats of group D were injected with 1000 gamma of iodide every 8 hours for 28 days, and in addition were fed the stock diet to which had been added 0.2 per cent propylthiouracil.

Uninjected, untreated rats maintained on the same stock diet were kept as normal controls (group A).

At the end of the experiment, all rats were anesthetized with sodium pentobarbital and exsanguinated. The entire neck region (soft tissues anterior to prevertebral fascia) was removed and fixed in 10 per cent neutral formalin. The pituitaries were fixed in sublimate-formal (9:1). The neck region was serially sectioned at 10 micra and stained with hematoxylin and eosin. The pituitaries were sectioned at 2–3 micra and stained by a modification of the method of Martins (1933). Pituitary cell counts were carried out as described earlier (Goldberg and Chaikoff, 1950a).

RESULTS

Microscopic examination of serial sections of the entire cervical region of all I^{131}-treated rats (groups B, C, and D, Table 1) revealed no recog-

TABLE 1. PERCENTAGE OF ACIDOPHILS IN THE ANTERIOR PITUITARY

Group†	Treatment	Acidophils Per cent of total cells*
A	Normal	44
B	Radiothyroidectomy	2
C	Radiothyroidectomy plus 1000 gamma iodide thrice daily	23
D	Radiothyroidectomy plus 1000 gamma iodide thrice daily plus 0.2 per cent propylthiouracil	3

* At least 2000 cells counted per gland.
† Ten rats per group.

nizable thyroid tissue. In these rats the thyroid glands were completely replaced by dense, fibrous tissue. Intact normal-appearing parathyroids were found in all rats. A detailed study of the histopathological changes that occur in the thyroid after radioiodine injections has already been reported by Goldberg *et al.* (1950b).

Acidophils were present to the extent of about 44 per cent in the anterior pituitaries of the normal, untreated rats (Table 1). This value is in good agreement with earlier observations made in this laboratory (Goldberg and Chaikoff, 1949; 1950a) and elsewhere (Griesbach and Purves, 1943).

Acidophils were almost completely absent in the anterior pituitaries of the rats in group B, which had been fed a stock diet containing about 0.6 gamma of iodine per gram (Table 1).[3]

[3] We are indebted to Mr. J. Meier for the iodine determinations.

Cytological examination of the anterior pituitaries of the radiothyroid ectomized rats that had been injected thrice daily with 1000 gamma iodide as KI (group C) disclosed that regranulation of the acidophils had occurred (Table 1). The granulated acidophils were present to the extent of about half the normal value.

The administration of propylthiouracil to the iodide-treated, thyroidectomized rats (group D) prevented the regranulation of the acidophils (Table 1); the cytological picture of the anterior pituitaries of group D was identical with that observed in the untreated, thyroidectomized controls (group B).

DISCUSSION

Several investigators have attempted to obtain evidence for extrathyroidal formation of thyroxine. Thus, in 1941, Chapman reported that oxygen consumption was higher in thyroidectomized rats maintained on a high iodine diet than in those maintained on a low iodine diet, and from this observation he was led to suggest that a thyroxine-like substance can be produced in extrathyroidal tissues. But as pointed out by Morton *et al.* (1943), it is questionable whether the small increases in oxygen consumption observed by Chapman can be accepted as evidence for the formation of extrathyroidal thyroxine. Moreover, the *complete* absence of thyroid tissues in his rats was not established. *It has been shown that about 50 per cent of rats subjected to surgical thyroidectomy still contain iodine-concentrating tissue* (*Reinhardt, 1942*).

Morton *et al.* (1943), with the aid of radioactive iodine, obtained evidence for the formation of a thyroxine-like substance in surgically thyroidectomized rats. The completeness of thyroidectomy was exhaustively tested 1) by examination of serial sections of the neck and chest regions and 2) by demonstrating the absence of iodine-concentrating tissues in the neck and mediastinal regions as measured by radioautographic procedures.

Dvoskin (1947) found that the subcutaneous injection of elemental iodine into thyroidectomized or goitrogen-treated rats produced thyroxine-like effects. His findings were confirmed, only in part, by Ferguson and Sellers (1949). According to Barker and Lipner (1948, 1949), direct iodination of tissue protein *at the site of the I_2 injection* could account for the observed effects.

Purves and Griesbach (1946) first observed that the injection of 1 mg. of iodine as iodide resulted in regranulation of the pituitary acidophils in *surgically thyroidectomized* rats. The observations recorded here show that excess iodide also regranulates the acidophils in rats that have been thyroidectomized by injections of radioiodine, a procedure shown to insure *total* destruction of all thyroid tissue including aberrant glandular tissue (Goldberg *et al.*, 1950b). It is of importance to note that this acidophilic

response to iodide is completely prevented by the feeding of substances like propylthiouracil and thiourea (Purves and Griesbach, 1946) which are known to inhibit the conversion of iodide to thyroxine (Franklin *et al.*, 1944). But the goitrogens have no effect on the acidophilic regranulation induced by thyroxine in thyroidectomized rats (Purves and Griesbach, 1946). Since thyroxine or metabolically active, structural isomers of thyroxine, such as chloro- or bromo-derivatives, are the only substances known to maintain the pituitary acidophils in the thyroidectomized rat, it is reasonable to conclude that iodide is converted to thyroxine in the rat totally devoid of thyroid tissue.

SUMMARY

1. The sensitive response of anterior pituitary acidophils to the presence of minute amounts of circulating thyroxine was utilized to detect thyroxine synthesis in the rat completely deprived of thyroid tissue by I^{131} treatment.

2. It is shown that the thrice daily injection of 1000 gamma of iodide results in regranulation of the pituitary acidophils.

3. Simultaneous administration of propylthiouracil completely prevents the acidophil response to iodide injections.

4. It is concluded that thyroxine can be synthesized in the rat totally devoid of thyroid tissue.

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CYTOCHEMICAL CHANGES IN THE ADRENAL CORTEX OF THE RAT IN PYRIDOXINE DEFICIENCY*

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HISTOLOGICAL changes observed in the adrenal cortex of pyridoxine-deficient rats, though suggestive of the alarm and adaptation reaction, have not been attributed to the absence of pyridoxine in the diet (Antopol and Unna, 1942). However, other cytochemical studies have demonstrated a transitory augmentation of lipoid in the adrenal cortex of rats fed a pyridoxine-free diet for three weeks (Deane and Shaw, 1947).

Malnutrition has been shown to effect a modification in structure and function of endocrine glands indicative of hypofunction (Jackson, 1925, 1929; Stephens, 1941). Morphologic changes in the adrenal gland are difficult to interpret, since differences in the degree of inanition as well as certain vitamin deficient states markedly influence the adrenal gland. Complete inanition generally produces hypertrophy, whereas, chronic underfeeding, at least in the rat, may result in atrophy of the adrenals (Mulinos and Pomerantz, 1941; Mulinos *et al.*, 1942; Cameron and Carmichael, 1946). Hence, inanition must be controlled to minimize ambiguity in the interpretation of morphologic changes in the adrenal cortex during the study of induced vitamin deficiencies.

The antagonistic action of certain vitamin analogs provides a useful approach to the problem of adrenal glandular function in the presence of vitamin deficiency and the cyto-physiological changes operating therein. Desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-methyl-pyridine), a pyridoxine analog, exerts strong antipyridoxine properties in the chick (Ott, 1946). The inner zones of the adrenal cortex of dogs and monkeys receiving this substance are depleted of sudanophilic lipoid (Mushett, Stebbins and Barton, 1947).

There are no published data concerning the cyto-chemistry of the adrenal cortex of the pyridoxine-deficient rat wherein the deficiency is accelerated by administration of the vitamin inhibitor, desoxypyridoxine. It therefore appeared desirable to compare the cytochemical changes in the

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adrenal cortices of pyridoxine-deficient rats in which the deficiency develops slowly by feeding a pyridoxine-free diet with deficiency accelerated by desoxypyridoxine. The extent to which inanition influences these changes was also studied.

MATERIALS AND METHODS

The rats used in these experiments were weanling albino males of the Carworth strain weighing 40 to 60 gm. The animals were housed individually in an air conditioned laboratory at a temperature of 75° F. and relative humidity of 50%.

The pyridoxine-free diet used in this study consisted of the following basic constituents: vitamin-free casein (Labco), 24%; dextrose, 60%; salt mixture, U.S.P. XI No. 1, 4%; Crisco, 10%; and cod liver oil, 2%. Each kilogram of this basic ration was supplemented with 10 mgm. of thiamine, 20 mgm. riboflavin, 100 mgm. each of nicotinamide and calcium pantothenate, 50 mgm. of inositol and 1 gm. choline. The complete purified diet[1] consisted of the above basic ration and micronutrients, to which was added 10 mgm. of pyridoxine per kilogram.

EXPERIMENTS

A comparison was made of the relative effects of pyridoxine-deficiency and a similar degree of inanition upon the adrenal gland. Pyridoxine-deficiency was accelerated by subcutaneous administration of desoxypyridoxine (6 mgm./100 gm./day). The animals were divided into five groups of ten rats each and maintained for three weeks as follows:

Group 1. Pyridoxine-free diet *ad libitum:* desoxypyridoxine.
Group 2. Pyridoxine-free diet; daily body weight individually restricted to those of rats in group 1.
Group 3. Complete purified diet; daily body weight individually restricted to those of rats in group 1.
Group 4. Pyridoxine-free diet; daily food intake individually restricted to those of rats in group 1.
Group 5. Complete purified diet; daily food intake individually restricted to those of rats in group 1.

Two or more animals were sacrificed at weekly intervals, from each group to determine the progressive morphological changes in the adrenal cortex.

In a second experiment a study was made of the relative effects of pyridoxine deficiency (induced without desoxypyridoxine) and of a comparable degree of inanition upon the adrenal gland. The rats were divided into four groups of fourteen animals each and maintained for five weeks as follows:

Group 1. Pyridoxine-free diet *ad libitum.*
Group 2. Complete purified diet; daily body weight individually restricted to those of rats in group 1.

[1] This ration, commonly employed in the Merck Institute, was prepared by the nutritional division, supervised by Dr. Gladys A. Emerson.

Group 3. Complete purified diet; daily food intake individually restricted to those of rats in group 1.
Group 4. Complete purified diet *ad libitum*.

Morphological changes were observed in the adrenal glands of two or more rats per group sacrificed weekly.

Finally, the effect of pyridoxine (6 mgm./100 gm.) on the adrenal cortex of deficient animals was investigated. Thirty-five rats were fed pyridoxine-free diet and injected daily for three weeks with desoxypyridoxine (6 mgm./100 gm.). Three procedures were used.

1. Five deficient animals were given single or repeated doses of pyridoxine subcutaneously, as follows: a) each of two rats received one injection and was sacrificed at 24 and 48 hours, respectively, b) single rats received two, three and four injections and each was sacrificed 24 hours after the last dose. For each depleted rat receiving pyridoxine, two control animals were maintained on pyridoxine-free diet and two were fed complete purified diet. The food intake of the control rats was regulated so that their body weight changes approximated those of the pyridoxine-dosed animals.

2. Twenty pyridoxine-deficient rats were unilaterally adrenalectomized and sacrificed after 24 hours. Ten of these animals received a single dose of pyridoxine immediately after operation.

TABLE 1. THE EFFECT OF PYRIDOXINE-DEFICIENCY ACCELERATED BY DESOXYPYRIDOXINE-TREATMENT ON ABSOLUTE WEIGHT OF ADRENAL GLAND

Group	Weeks on diet	No. rats	Body weight in grams		Adrenal weight in grams	F
			Initial	Final		
1						
Defic. Diet	1	2	48	48	0.015	
and desoxy-	2	2	47	48	0.023	
pyridoxine	3	6	51	50	0.031	
2						
Controls	1	2	48	46	0.016	
Defic. diet	2	2	47	48	0.020	
Pair-Weight	3	6	53	54	0.021	29.1*
3						
Controls	1	2	48	48	0.016	
Compl. diet	2	2	47	50	0.017	
Pair-Weight	3	6	52	52	0.019	86.1*
4						
Controls	1	2	48	50	0.017	
Defic. diet	2	2	47	67	0.019	
Pair-fed	3	6	51	78	0.019	35.5*
5						
Controls	1	2	49	51	0.016	
Compl. diet	2	2	47	64	0.020	
Pair-fed	3	6	51	82	0.020	18.8*

F (1, 9) = 5.12 for p = 5%; = 13.6 for p = 0.5%.
* Highly significant source of variation.

TABLE 2. THE EFFECT OF PYRIDOXINE-DEFICIENCY WITHOUT DESOXYPYRIDOXINE-TREATMENT ON ABSOLUTE WEIGHT OF ADRENAL GLAND

Group	Weeks on diet	No. rats	Body weight in grams		Adrenal weight in grams	F
			Initial	Final		
1	1	2	58	79	0.016	
Defic. diet	2	2	57	80	0.021	
ad libitum	3	2	59	84	0.022	
	4	2	60	95	0.025	
	5	6	56	67	0.032	
2	1	2	58	76	0.017	
Controls	2	2	57	80	0.017	
Compl. diet	3	2	60	86	0.021	
Pair-Weight	4	2	60	93	0.020	
	5	6	57	69	0.021	32.1*
3	1	2	58	76	0.018	
Controls	2	2	57	93	0.021	
Compl. diet	3	2	60	95	0.021	
Pair-fed	4	2	60	110	0.020	
	5	6	57	90	0.020	28.5*
4	1	2	58	86	0.021	
Controls	2	2	57	132	0.023	
Compl. diet	3	2	58	138	0.024	
ad libitum	4	2	60	162	0.026	<1†
	5	6	58	203	0.032	

F (1, 9) =5.12 for p=5%; =13.6 for p=0.5%.
* Highly significant source of variation.
† Not significant.

3. Ten pyridoxine-deficient rats were dosed with pyridoxine until the acrodynia disappeared. During this time, the administration of desoxypyridoxine was continued. The animals were then sacrificed.

CYTOCHEMICAL METHODS

After mild chloroform anesthesia, the animals were exsanguinated. The paired adrenals were weighed, immersed in fixative and in addition frozen sections of adrenal glands were cut 15 μ thick.

The Deane and Morse modification (1948) of the Barnett and Bourne method (1941) was used to study the amount and distribution of ascorbic acid. Cholesterol was viewed in frozen sections after precipitation with digitonin (Windaus, 1909, 1910). Lipids were localized in frozen sections by means of Sudan IV and hematoxylin. Sections were observed with polaroid lenses for acetone-soluble substances exhibiting sterol-like birefringence (Dempsey and Bassett, 1943). The presence of aldehydes was detected by means of Schiff's reagent (Feulgen and Voit, 1942). The distribution pattern of sulfuric acid positive compounds indicative of biologically active steroids (Dempsey and Bassett, 1943) was studied. The Albert and Leblond modification (1946) of the Bennett method (1940)

was used to demonstrate the presence of substances generally considered carbonyl lipid phenylhydrazones.

STATISTICAL ANALYSIS

By applying the analysis of covariance technique (Snedecor, 1946) to the 6 adrenal weights at the 3rd week (dependent variate) and the corresponding 6 final body weights (independent variate), shown in Table 1, the observed adrenal weight variations were segregated into two portions, (a) the observed effect of treatment on the mean adrenal weights after adjustment to a common body weight and (b) the error of observation. The ratio of the treatment mean square divided by the error mean square (=F) for any pair of treatments i.e. group 1 compared with group 2, 3, 4, or 5 is related to the possibility that the difference could have occurred by chance. A probability of less than 5% was considered to be a significant source of variation. A similar procedure was followed with the adrenal weight-body weight data at the 5th week (Table 2).

RESULTS

Significant enlargement of the adrenal gland occurred only after feeding the pyridoxine-free diet when the vitamin antagonist desoxypyridoxine was given concomitantly and sooner than after caloric restriction of diet (Tables 1, 2).

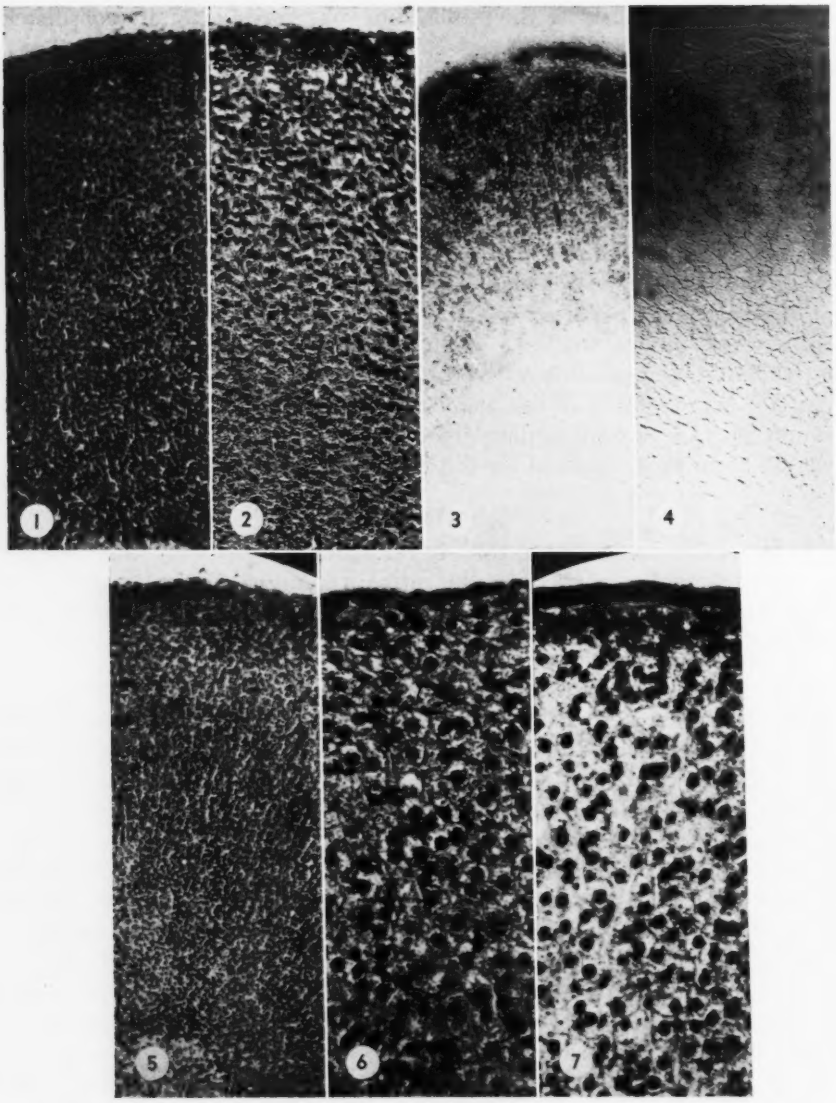
The adrenal cortex in control rats

The adrenal cortex was normal in control animals. The amount and distribution of lipoid-soluble droplets (Fig. 1), aldehyde-containing substances (Fig. 3), reduced silver particles indicative of ascorbic acid (Fig. 6), sterol substances in fat (Fig. 8) and cholesterol (Fig. 10) are shown photomicrographically. The cells of the adrenal cortex stained intensely with dinitrophenylhydrazine indicating the presence within the lipoid globules of carbonyl groups presumably ketosteroid substances. These cells also formed reddish-brown droplets or granules of variable size upon treatment with sulfuric acid, perhaps of a sterol-like nature.

The adrenal cortex in the deficient rats

Experiment 1

The acceleration of pyridoxine deficiency by administering desoxypyridoxine resulted in cytochemical changes in the adrenal cortex at two weeks. At three weeks, the adrenal cortex displayed a further loss in staining capacity. Sudanophilic material (Fig. 2) and birefringent particles (Fig. 9) were observed in appreciable amounts only in the glomerulosa. The Schiff reaction was decidedly reduced in the zona fasciculata (Fig. 4).



DESCRIPTION OF PLATE 1

All photomicrographs are of rat adrenal glands that were fixed in either 10% neutral formalin or acid alcohol-silver nitrate. Tissues shown in figures 1, 2, 3, 4 and 5 (×108) were sectioned on freezing microtome (15 μ) and mounted in glycerin gelatin; those in figures 6, 7 (×473) were embedded in paraffin and sectioned on rotary microtome (5 μ) and mounted in Clarite.

Cholesterol was barely demonstrable in this zone and was finely distributed in the glomerulosa (Fig. 11). Sections treated with sulfuric acid or dinitrophenylhydrazine showed a marked decrease of staining intensity in the fasciculata. Fewer particles of reducing substance (ascorbic acid) were evident in the cortices of deficient animals (Fig. 7).

Experiment 2

Rats fed pyridoxine-free diet showed morphological changes in the cortex at the third and fourth week that were comparable to those observed in Experiment 1 at the end of two weeks. At the fifth week, sudanophilia was virtually absent from the fasciculata (Fig. 2); the lipoid of the fasciculata and reticularis did not possess strong ketosteroid qualities and fewer particles of ascorbic acid were observed in the cells of the adrenal cortex. These changes were similar to those recorded in Experiment 1 at three weeks (Figs. 2, 4, 7, 9 and 11).

Experiment 3

1. A significant amount of cholesterol and sudanophilic material was observed in the cells of the fasciculata (Figs. 12, 5) after administering a single dose of pyridoxine to severely pyridoxine-deficient rats (antivitamin-treated). After four injections of pyridoxine, the distribution of lipoid and cholesterol was essentially normal.

FIG. 1. Control rat, body-weight restricted and fed complete purified diet 3 weeks. Sudan IV and hematoxylin. Lipoid droplets are densely packed in cells of glomerular zone, partially absent from transitional zone, paler staining in outer and deeper-staining in inner zone of fasciculata.

FIG. 2. Pyridoxine-deficient rat, antivitamin-treated and fed pyridoxine-free diet 3 weeks. Sudan IV and hematoxylin. The glomerulosa appears essentially normal; sudanophilia is virtually absent from inner zones of cortex.

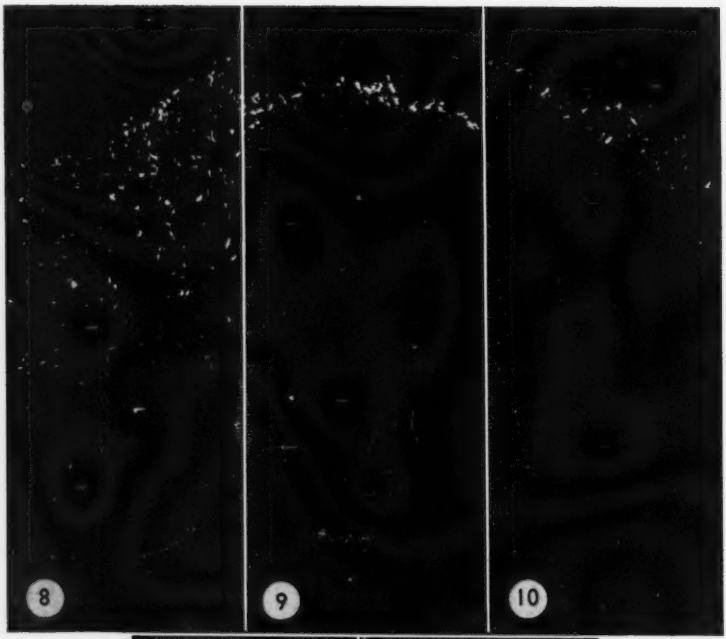
FIG. 3. Control rat, body-weight restricted and fed complete purified diet 3 weeks. Schiff plasmal reaction. Normal amount and distribution of Schiff-positive material is present in cortex.

FIG. 4. Pyridoxine-deficient rat, antivitamin-treated and fed pyridoxine-free diet 3 weeks. Schiff plasmal reaction. Reaction is slightly reduced in glomerular zone and is faintly visible in outer and absent from inner fasciculata.

FIG. 5. Pyridoxine-deficient rat, antivitamin-treated and fed pyridoxine-free diet 3 weeks. Sudan IV and hematoxylin. Replacement of lipoid 24 hours after unilateral adrenalectomy and single injection of pyridoxine. Cells of fasciculata show moderate amounts of sudanophilia.

FIG. 6. Control rat, body-weight restricted and fed complete purified diet 3 weeks. Hematoxylin and eosin. Granules of precipitated silver signifying ascorbic acid are distributed throughout cortex.

FIG. 7. Pyridoxine-deficient rat, antivitamin-treated and fed pyridoxine-free diet 3 weeks. Hematoxylin and eosin. A general decrease of precipitated silver (ascorbic acid) occurs in inner cortex.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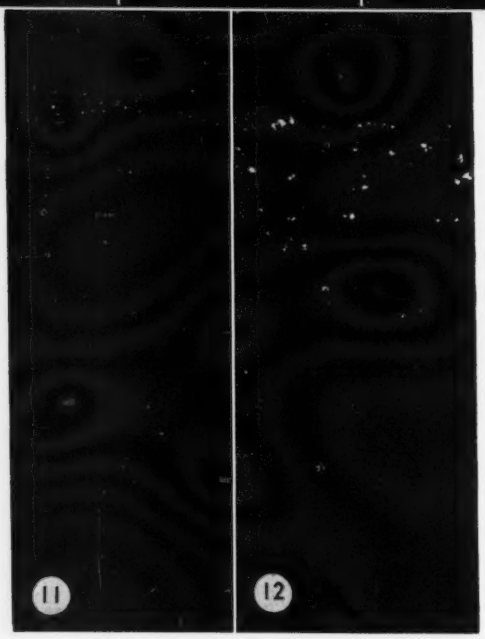
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2. The administration of a single dose of pyridoxine, to pyridoxine-deficient rats, immediately after unilateral adrenalectomy, also effected a significant repletion (i.e. within 24 hours) of cholesterol (Fig. 12) and sudanophilic material (Fig. 5) in the zona fasciculata. The cortices of unilaterally-adrenalectomized control rats showed no replacement of sudanophile or ketosteroid material.

3. Pyridoxine-deficient rats, receiving pyridoxine concomitantly with antivitamin showed a complete reversal of the signs of dermatitis. The adrenal parenchyma of these animals resumed an essentially normal appearance.

DISCUSSION

In the rat adrenal, hypertrophy is associated with a deficiency of certain factors of the vitamin B complex, such as thiamine (Deane and Shaw, 1947) and pantothenic acid (Deane and McKibbin, 1946). On the other hand, neither riboflavin nor pyridoxine deficiency is believed to cause more than a transitory stimulation of the adrenal cortex in the rat (Deane and Shaw, 1947). In the present study a distinct effect of pyridoxine deficiency on adrenal cortical tissue was demonstrated. The obvious disagreement between previous findings and the current results is most probably due to the difference in severity of the disease.

The administration of desoxypyridoxine accelerated the appearance of the deficiency symptoms and the morphological changes in the adrenal cortex. Comparable inanition produced no significant changes in the adre-

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DESCRIPTION OF PLATE 2

Photomicrographs (×108) are of rat adrenal glands that were fixed in 10% neutral formalin sectioned on freezing microtome (15 μ) and mounted in glycerin gelatin.

FIG. 8. Control rat, body-weight restricted and fed complete purified diet 3 weeks. Mounted unstained and observed with polaroid lenses. Many coarse and fine birefringent particles indicative of sterol substances occur in glomerulosa and fasciculata.

FIG. 9. Pyridoxine-deficient rat, antivitamin-treated and fed pyridoxine-free diet 3 weeks. Mounted unstained and observed with polaroid lenses. Birefringent material is maintained in glomerulosa, but markedly depleted in fasciculata.

FIG. 10. Control rat, body-weight restricted and fed complete purified diet 3 weeks. Reacted with digitonin, stained with Sudan IV and observed with polaroid lenses. The birefringent cholesterol particles are normally distributed.

FIG. 11. Pyridoxine-deficient rat, antivitamin-treated and fed pyridoxine-free diet 3 weeks. Reacted with digitonin, stained with Sudan IV and observed with polaroid lenses. Fine particles of cholesterol occur in glomerulosa; scant amount of cholesterol in fasciculata.

FIG. 12. Pyridoxine-deficient rat, antivitamin-treated and fed pyridoxine-free diet 3 weeks. Reacted with digitonin, stained with Sudan IV and observed with polaroid lenses. Replacement of cholesterol 24 hours after unilateral adrenalectomy and single injection of pyridoxine. Zona fasciculata shows moderate amount of cholesterol.

nal cortex. Thus, the extensive changes observed in the cortex of pyridoxine-deficient animals must be attributed to the effects of pyridoxine deprivation.

Several possible mechanisms may be suggested regarding the mode of action of pyridoxine deficiency on adrenal cortical structure and function. One is related to the possibility that the lack of pyridoxine creates an especially severe state of general stress and consequently produces the well-known pituitary-adrenal response. By the same mechanism various infections are known to produce similar adrenal changes (Tepperman, Engle and Long, 1943). The nutritional dermatitis characteristic of pyridoxine deficiency in rats may be complicated by superimposed cellulitis due to bacterial invasion (Antopol and Unna, 1942). Also, such animals display a lowered resistance (Stoerk, Eisen and John, 1947) which may lead to intercurrent diseases (Stoerk, 1948). It could therefore be that the adrenal changes in pyridoxine deficiency are secondary to invasion by microorganisms.

Another possible mechanism would involve a direct effect of pyridoxine on the steroid metabolism of the adrenal cortex. It seems just as reasonable to assume that the lack of this vitamin results in a reduced capacity of the cortical tissue to manufacture its hormone. The dramatic ability of pyridoxine to restore an almost normal lipoid pattern in the adrenal cortex in a short time (i.e., within 24 hours) strongly supports this assumption.

The present studies obviously should be extended to nutritional diseases of comparable severity (by means of other vitamin antagonists) so that the specificity of the presently observed effect may be ascertained.

SUMMARY AND CONCLUSIONS

Evidence has been presented to suggest that severe pyridoxine deficiency causes morphological alterations in the adrenal cortex.

Significant enlargement of the adrenal gland of pyridoxine-depleted rats, predominantly involving the zona fasciculata, was observed when severe pyridoxine deficiency was produced with the vitamin antagonist, desoxypyridoxine. This effect of pyridoxine deprivation was not a consequence of caloric restriction.

The microscopic appearance of the adrenal cortex was greatly altered in pyridoxine-deficiency. The zona fasciculata showed a progressive loss, to the point of extinction, of sudanophilic lipoid, birefringent material and cholesterol. In sections treated with dinitrophenylhydrazine, sulfuric acid or Schiff's reagent, a comparable decrease in affinity for stain was found in this zone. The amount of ascorbic acid-like material in the cells of the adrenal cortex was reduced in deficient animals. The administration of a single dose of pyridoxine to pyridoxine-depleted rats receiving desoxy-

pyridoxine produced a rapid restoration of lipoid and cholesterol in the zona fasciculata.

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EFFECT OF A SINGLE INJECTION OF DIETHYLSTILBESTROL ON THE ADRENAL CORTEX IN THE GOLDEN HAMSTER

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THE pituitary gland of the hamster shows marked hyperplasia and adenoma formation following chronic treatment with estrogens (Vasquez-Lopez, 1944; Koneff, Simpson and Evans, 1946). More recently, Mathews, Kirkman and Bacon (1947) reported that, with diethylstilbestrol, malignant renal tumors appear also, in practically all male, but not female, hamsters adequately treated. Females tend to show kidney damage similar to that found in glomerulo-nephritis. Since similar renal damage, occurring as part of the adaptation syndrome, is known to be secondary to adrenal cortical stimulation, it is considered possible that stilbestrol may stimulate the pituitary gland to release adrenocotrophin (ACTH) which in turn may stimulate the adrenal cortex to discharge certain steroids which may participate in these kidney changes. In order to determine whether ACTH is released, a single injection of diethylstilbestrol was given to both male and female hamsters and their adrenals analyzed according to various chemical and histochemical methods indicative of ACTH-release.

GENERAL METHODS

Hamsters (Cricetus auratus) between 90 and 100 days old were divided into two separate groups according to sex. Each animal was given an intraperitoneal injection of 0.06 mg. of diethylstilbestrol (Lilly) in $\frac{1}{4}$ c.c. of cottonseed oil and sacrificed by decapitation at various time-intervals: females, 0, $\frac{1}{3}$, 1, 3, 6, 12, 24, 36, and 48 hours; males, at 0, $\frac{1}{3}$, 1, 3, 6, 12, 24, 48, and 96 hours. At the time of sacrifice adrenal glands were removed as quickly as possible, trimmed free of fat, weighed to the nearest milligram on a Roller-Smith torsion balance, placed in cold 2.5% metaphosphoric acid, homogenized, and analyzed in a Coleman spectrophotometer for their ascorbic acid content according to the method of Roe and Keuther (1943). Each homogenate contained a pool of one adrenal from each of two animals, the remaining two adrenals from these animals being placed in either 10% neutral formalin or 10% $AgNO_3$ for histochemical and histological analyses. In the case of the silver nitrate method the author employed dark bottles containing equal parts of 10% acetic acid and 10% $AgNO_3$ for fixation and the precipitation of ascorbic acid; this was followed by a 5% acid hypo solution, a rinse with water, and sectioning at 10 micra on the freezing microtome.

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RESULTS

Chemical analysis for adrenal ascorbic acid in the female hamster showed an abrupt fall within 30 minutes, and a maximum of depletion within one hour. The ascorbic acid content then began to increase so that by 6 hours it was much above normal, returning to normal 36 hours after the initial injection (Figure 1 and Table 1). These results are very similar to those of Sayers and Sayers (1948), who assayed the potency of ACTH by its ability

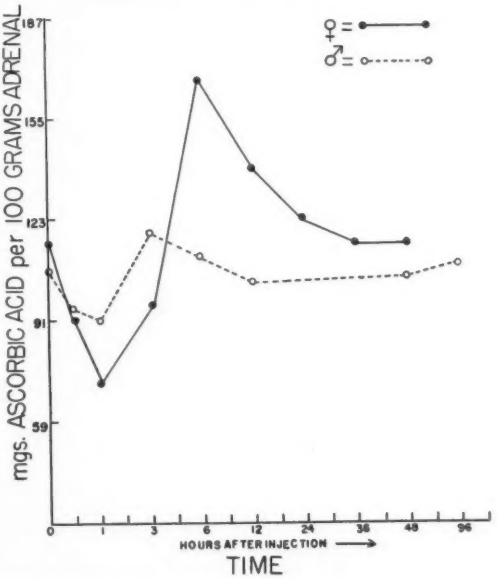


FIG. 1. Changes in male and female hamster adrenal ascorbic acid following a single intraperitoneal injection of 0.06 mg. of stilbestrol.

to cause the depletion-repletion of adrenal ascorbic acid in hypophysectomized male rats.

Surprising results were obtained when this same regimen of injection and sacrifice was applied to the male hamster. In this sex there seems to be no sudden marked release of ACTH as in the female. Instead, there is only a slight fall in the adrenal ascorbic acid during the first hour. After 3 hours the ascorbic acid has returned to slightly above normal, but after 12 hours it has dropped slightly below normal again, requiring 96 hours recuperation-time before returning to normal (Figure 1 and Table 1).

When the differences between the arithmetic means and their standard errors for the ascorbic acid changes of all male and female groups were analyzed by the method of Palmer (1934) for testing the validity of the

response-to-treatment of each sex, the p value was 0.0006, i.e. highly significant. This definite sex difference in the response of adrenal ascorbic acid to stilbestrol suggests that in this species there may exist a corresponding difference in the effect of stilbestrol on ACTH-release.

Many authors have reported adrenal enlargement in the rat following stimulation with ACTH and exposure to stress. After subjecting both male and female hamsters to the treatment described in this experiment, there were great changes in both male and female adrenal weights, and all within 48 hours time (Table 1).

TABLE 1. CHANGES IN FEMALE AND MALE HAMSTER ADRENAL ASCORBIC ACID AND IN THE RATIO OF ADRENAL WT./BODY WT. AT VARIOUS INTERVALS FOLLOWING A SINGLE INJECTION OF 0.06 MG. OF DIETHYLSTILBESTROL

Time (hrs.)	No. of animals	Average adrenal weight	Ratio: Adrenal wt./body wt. ×1000	p value	Mgs. ascorbic acid per 100 gms. tissue with S.E.M.	p value
			Female			
0	16	8.9	67 4.0	*	115± 2.1	*
½	8	8.4	76 3.2	.0801	92± 3.8	.0001
1	8	9.3	74 1.8	.1906	71± 1.2	.0001
3	8	11.0	85 3.6	.0008	95± 3.7	.0001
6	8	10.3	94 3.8	.0001	165±17.4	.0044
12	8	8.5	73 2.1	.1835	132±10.4	.1096†
24	16	9.5	83 2.1	.0004	124±16.3	.5823†
36	8	8.7	70 3.2	*	114± 3.1	*
48	8	8.5	59 3.9	.1527	114± 4.8	*
			Male			
0	16	12.9	110 2.6	*	109± 2.1	*
½	12	12.8	108 3.5	*	95± 2.4	.0001
1	20	12.5	104 2.3	.0854	92± 1.1	.0001
3	12	13.1	113 3.4	*	117± 3.9	.0719
6	8	12.2	108 3.7	*	111± 3.7	*
12	8	13.6	128 4.9	.0012	97± 1.3	.0012
48	8	12.3	105 3.6	.2627	98± 2.0	.0002
96	4	10.4	95 7.7	.0658	112± 9.0	*

* This value is equal to normal and cannot be compared statistically.
† This value is not significant. (This is probably due to the fact that there is much individual variance amongst animals in their ability to resynthesize ascorbic acid.)

Frozen sections cut at 10 μ were made of those adrenals which had been treated with acidified $AgNO_3$. In the normal unstimulated female hamster the adrenals showed a cortex in which the black silver granules indicative of ascorbic acid were very fine and evenly dispersed throughout the cytoplasm of cells in the zona glomerulosa, fasciculata, and reticularis. There was no evidence of ascorbic acid in the medullary sinusoids. The capillaries of the glomerulosa and outermost fasciculata were filled with these granules serving to display the capillary network in this area (Figures 2 and 3).

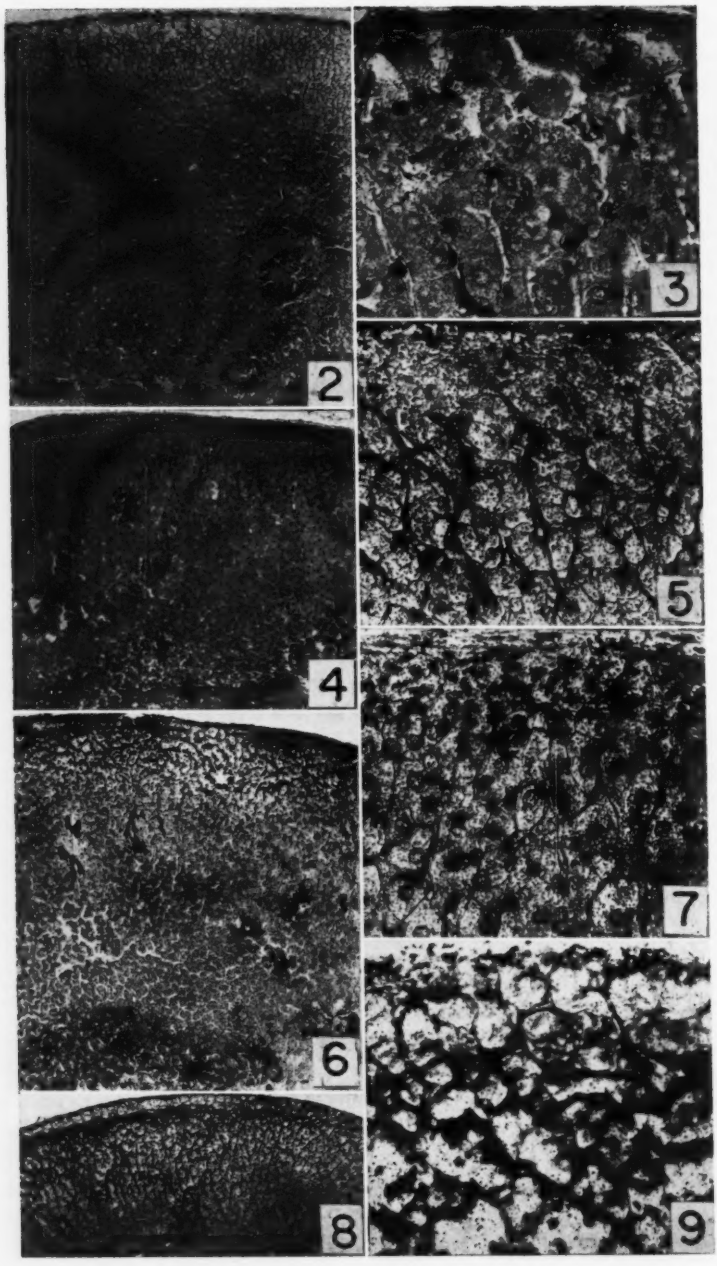
In the adrenal of a female hamster given a single injection of stilbestrol and sacrificed 1 hour later (Figures 4 and 5), the ascorbic acid granules were coarser and the capillary reticulum extended farther into the zona fasciculata. In the adrenal of an animal of the same sex sacrificed six hours later, the granules were much coarser, the vascular network extended well into the fasciculata, and the ascorbic acid granules were beginning to appear in the sinusoids of the medulla (Figures 6 and 7). In the adrenal of an animal allowed to go 48 hours, the granules were coarse, the mesh of engorged capillaries extended from glomerulosa through reticularis, and the sinusoids of the medulla were full of granules (Figures 8 and 9). In some cases where the central vein could be seen, its lumen was filled with these granules.

Sections of male hamster adrenals stained for ascorbic acid showed no such marked differences. Adrenal sections from males in groups that chemically were maximally depleted and those that were maximally repleted were practically identical. The ascorbic acid granules in both cases were fine and evenly dispersed, and the capillary reticulum in both cases extended to mid-fasciculata (Figures 10 and 11).

Those adrenals which had been placed in 10% neutral formalin were cut at 15 μ on the freezing microtome and tested by the Schiff, Schultz cholesterol, Seligman, and sudan orange methods. With the Schiff reagent no changes in plasmalogen were detected in hamsters of either sex. The entire cortex in both sexes was Schiff-positive (Figure 12). The Seligman test disclosed one notable fact, not shown by any of the other histochemical tests, i.e. that there is a marked lipid-free zone in the female hamster and only a suggestion of such a zone in the male. This lipid-free zone did not increase appreciably in width at any time during the course of the experiment in either sex (Figures 13 and 14). The Schultz cholesterol test was negative in the adrenals of both sexes throughout the course of the experiment. The lipid stain, sudan orange, used for some sections, colored the lipid-droplets of the cortex pale orange.

DISCUSSION

In the hamster the marked sex difference in depletion-repletion pattern of response of adrenal ascorbic acid to stimulation is very striking. The curve (Figure 1) for the female hamster is similar to that found in the rat and the guinea pig as reported by Long *et al.*, 1947 and Sayers and Sayers, 1948. From our experience in this laboratory (Kirkman and Bacon, 1950) e.g., the induction of tumors in males, and a tendency for nephritis to develop in females, after prolonged treatment with stilbestrol, we anticipated the sex difference in adrenal ascorbic acid response, described above. This raises the question as to whether or not there is a blocking mechanism for ACTH-release associated with the testis. Selye (1950) maintains that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testosterone is capable of blocking the release of endogenous ACTH, but not exogenous ACTH. Parenthetically, it is interesting to point out here that this situation is in keeping with the behavior of the male and female hamster, *i.e.* the female is much more hardy and aggressive than the male. Perhaps this is so because of the greater responsiveness of the female adrenal and/or pituitary gland imparting to the sex a greater capacity for adaptation and resistence to stress.

Since the female hamster does appear to release ACTH when given a single injection of stilbestrol, with continued administration of this estrogen there might be a continuous stimulation of the pituitary gland to release ACTH. This would cause a continuous release of adrenal corticoids, including such steroids as desoxycorticosterone, continuous dosage with which is known to produce nephritis and hypertension in rats (Grollman, Harrison and Williams, 1940; Swingle, Parkins and Remington, 1941). Grollman *et al.* have also produced hypertension and renal damage in rats with stilbestrol. The symptoms produced by them were claimed to be similar in all respects to those produced by chronic treatment with DOCA. Many of our female hamsters treated with stilbestrol developed nephritis; however, the hamster seems to be naturally susceptible to this disease, since we have found nephritis in a few untreated female hamsters.

The sections showing ascorbic acid in the hamster adrenal following a single stilbestrol injection (Figures 2 to 12) are consistent with the marked changes demonstrated by the chemical method of analysis. The changes in the size of ascorbic acid granules and the changes in the pattern of capillary distension and distribution indicate an attempt to replete the adrenal cortex with its normal content of vitamin C. In the female this is most marked after 48 hours when the adrenal has returned to its chemically-demonstrable normal content of vitamin C. In the Long-Evans rat the histochemical and chemical demonstration of ascorbic acid changes in the adrenal cortex is of a different pattern. Histochemically, there are the

FIGS. 2 and 3. Adrenal cortex of a normal female hamster. Note the capillary reticulum in the zona glomerulosa and outer fasciculata. The granules of ascorbic acid (black) cause the capillary pattern to stand out. In fig. 3 the granules are very fine. 10% $AgNO_3$; fig. 2, ×50, fig. 3, ×400.

FIGS. 4 and 5. Cortex of female 1 hr. after i.p. inj. of stilbestrol. Note extension of capillary reticulum farther into z. fasic. in fig. 4. In fig. 5 granules have become slightly coarser than in fig. 3. 10% $AgNO_3$; fig. 4, ×50, fig. 5, ×400.

FIGS. 6 and 7. Cortex of female 6 hours after initial inj. of stilb. Capillary net extends further into cortex than in fig. 4. In fig. 7 the granules have become even more coarse 10% $AgNO_3$; fig. 6, ×50, fig. 7, ×400.

FIGS. 8 and 9. Female adrenal 48 hrs. after inj. of stilb. The capillary net now extends the full length of the cortex from z. glom. to z. retic. Fig. 9 shows granules of a coarse texture and engorged capillaries. 10% $AgNO_3$; fig. 8 ×50, fig. 9, ×400.

same changes in size and distribution of ascorbic acid granules, but there is almost complete disappearance of granules after 3 hours with a maximal distribution and increase in granule-size at 12 hours, and a return to normal by 24 hours (Wexler, 1948). Also, there is no marked capillary engorgement by granules as is found in the hamster.

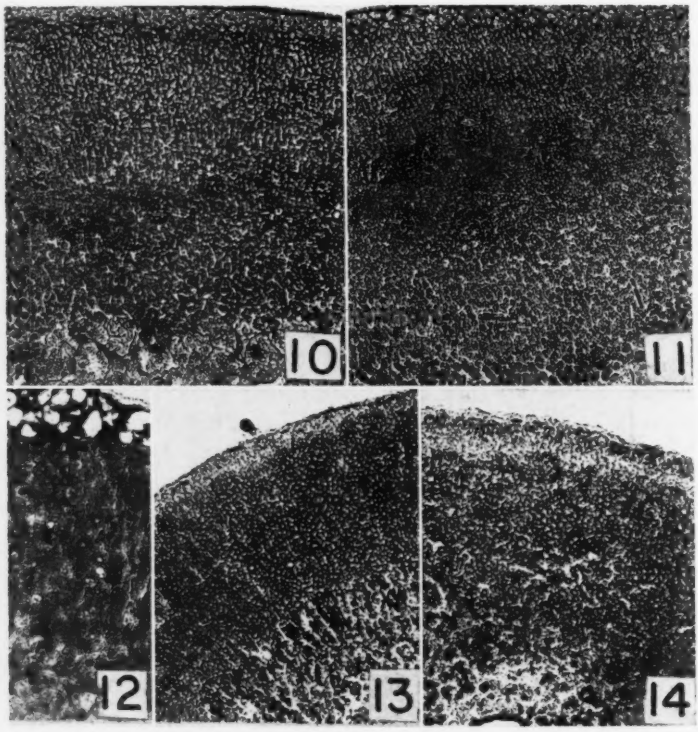

FIG. 10. Adrenal cortex of male hamster at the height of ascorbic acid depletion. Note capillary reticulum extends well into z. fasic. 10% $AgNO_3$; ×50.

FIG. 11. Cortex of male hamster at height of repletion of ascorbic acid. The capillary reticulum is almost identical with that of fig. 10. Because of the slight changes taking place in male adrenal ascorbic acid, very little change could be discerned in any male group. 10% $AgNO_3$; ×50.

FIG. 12. Cortex of female at height of stilb. stimulation (6 hrs.) no lipid or ketosteroid changes discernible. Also, absence of any lipid-free zone. Contrast this with figs. 13 and 14. Schiff test; ×50.

FIG. 13. Normal male adrenal cortex treated with Seligman reagent. Although entire cortex reacts positively, a sudanophobic zone appears. This is narrow in the male adrenal cortex, ×50.

FIG. 14. Cortex of female. Note wide lipid-free zone compared to that of male in fig. 13. The width of this zone did not change during the course of the experiment. Seligman test; ×50.

That there are no demonstrable changes in lipid content is in keeping with what has been found by others, *i.e.* that stimulation of much greater intensity and duration must be given in order to produce adrenal cortical lipid-alterations than is necessary to produce ascorbic acid alterations (Fortier, Skelton, Constantinides, Timircas, Herlant and Selye, 1950; Wexler, 1948). Moreover, the lipids present in the adrenal cortex of the hamster do not stain well with the various lipid stains available (Peczenick, 1944; Koneff *et al.*, 1946). The zona glomerulosa of the hamster seems to be especially poor in lipid material, whereas in the rat it is this zone which shows up especially well with lipid stains. Deane and Greep (1947) have suggested that the zona glomerulosa is responsible for the synthesis of desoxycorticosterone-like hormones and the fasiculata for the carbohydrate regulating hormones. The lipid-poor zona glomerulosa in the hamster and the frequency of nephritic conditions found in this animal adds further weight to their argument.

The negative Schultz cholesterol reactions found in this experiment are consistent with those reported by Alpert (1950) in hamsters treated longer with heavier doses. The appearance of a negatively reacting zone (the "sudanophobic" zone of others) at the juxta-glomerulo-fascicular region in sections subjected to the Seligman test (Figures 13 and 14), but not in sections treated with the Schiff reagent, adds an interesting note to the controversy as to the specificity of these two histochemical tests (Dempsey and Wislocki, 1946). This zone is broader in the female hamster than in the male.

Since the effect of stilbestrol on adrenal ascorbic acid in the hamster strongly suggests a pituitary gland involvement, it is our intention to investigate the possible application of this information to our growing understanding of the mechanisms involved in the induction of renal tumors and of nephritis in male and female hamsters following chronic treatment with this estrogen.

SUMMARY

A single intraperitoneal injection of 0.06 mg. of stilbestrol was given to groups of male and female hamsters and the animals sacrificed after the expiration of the following time intervals: 0, $\frac{1}{3}$, 1, 3, 6, 12, 24, 36, and 96 hours. Chemical analyses of adrenal glands for ascorbic acid showed changes suggesting a release of ACTH in the female hamster, but only a moderate response in the male. Various histochemical and histological tests failed to show any alteration in adrenal lipid constituents.

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NEURO-ENDOCRINE AND ENDOCRINE INFLUENCES ON THE CIRCULATING BLOOD ELEMENTS

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INTRODUCTION

THE complete blood count in patients with endocrine disorders frequently shows definite alterations from the normal pattern of circulating blood elements. Thus, in Cushing's syndrome due to either a lesion primary in the hypothalamus or to a tumor of the adrenal cortex, polycythemia, neutrophilia, lymphopenia and eosinopenia are frequent findings (De La Balze, *et al.*, 1946). On the other hand, in Addison's disease anemia, lymphocytosis and neutropenia are rather characteristically found (De La Balze, Reifenstein and Albright, 1946, *loc. cit.*; Baez-Villasenor, Rath and Finch, 1948). Reference to these two endocrine disorders is made because Cushing's syndrome is associated with adrenal cortical overaction while Addison's disease is an expression of adrenal cortical hypofunction. In diseases of the thyroid gland, hypofunction may be associated with anemia (Minot, 1921; Bistrom, 1946). The white blood count may be elevated slightly but the differential count usually is not changed. Hyperthyroidism is associated with a normal red count. The white blood count may be low or normal. There may be a relative neutropenia and a lymphocytosis. Along with the lymphocytosis there may occur hyperplasia of the lymphoid organs of the body. An extensive review of the blood findings in the various endocrine disorders has been made recently by Daughaday, Williams and Daland (1948).

The influence of the endocrine glands on the circulating blood elements of the dog may be studied experimentally with fewer complicating factors than are present in the hospital patient. The work of Harlow and Selye (1937), of Dougherty and White (1944) and others has established that in mice, in the rat, and in man the pituitary-adrenal hormone complex exerts a modifying influence on the circulating lymphocytes, eosinophiles and neutrophiles, and also on the amount of thymic and lymphoid tissue. With overactivity of the pituitary-adrenal hormone complex the circulating

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neutrophile count rises while those of the circulating lymphocytes and eosinophiles are depressed. Underactivity of the complex leads to an opposite effect in all respects.

In this paper are presented the effects in the dog of various alterations in neuro-endocrine and endocrine interrelations of long duration on the circulating blood elements, the thymus and the lymphoid tissue of the body. The alterations include those effected by both partial and complete denervation of the neural hypophysis, by simple hypophysectomy, bilateral adrenalectomy, total thyroidectomy and gonadectomy. Observations have been made on the effect of administering various hormone fractions to the dogs before and after these various alterations in their endocrine constitution. There are included also studies on the effect of exogenous epinephrine on the circulating blood elements. A mechanism of epinephrine action in its release of adrenocorticotropic hormone is offered. By this mechanism epinephrine is regarded as stimulating the cerebral cortex which in turn releases impulses which ultimately are inhibitory to those hypothalamic nuclei which innervate the neural hypophysis. The resultant depression of the secretion of the neural hypophysis permits the glandular hypophysis to release adrenocorticotropic hormone at an increased rate (Heinbecker and Pfeiffenberger, 1950).

EXPERIMENTAL MATERIALS AND METHODS

Dogs were used as the experimental animals in our studies of the circulating blood pattern. They were kept on a standard diet of Purina dog chow with meat added twice a week. Young dogs and dogs just received from the pound were wormed well in advance of the studies.

The operations carried out on the dogs consisted of (1) simple hypophysectomy through the oral approach, (2) complete and partial denervation of the neural hypophysis through the oral approach (plate 1-A), (3) bilateral adrenalectomy, (4) total thyroidectomy, (5) gonadectomy and (6) gonadectomy plus total thyroidectomy.

The exogenous hormone preparations administered to these dogs were preloban, ACTH, epinephrine, growth hormone, whole posterior pituitary extract, cortisone, desiccated pork thyroid gland substance, pituitary thyrotropic hormone and several combinations of two of the aforementioned hormone preparations.[2] The dosages used are

[2] The hormones used in these experiments were supplied through the courtesy of the following: ACTH, Thyrotropin and Growth Hormone by the Biochemical Research Division, Armour and Company, Chicago, Illinois. The ACTH, Lot 60–61, was stated to have a biological potency of 100 per cent of Armour Standard La-I-A and an oxytocin content of 0.01 units per mg. The growth hormone, Lot 22KR1, was stated to have a potency equivalent to Lot 22KR2 which has been accepted as the temporary standard by the National Institutes of Health. The Preloban Niphanoid was supplied by Winthrop Chemical Company, New York, New York, the posterior lobe extract by the Eli Lilly Company, Indianapolis, Indiana, and the cortisone by Dr. J. M. Carlisle, Medical Division, Merck and Company, Rahway, New Jersey. The epinephrine used was adrenalin chloride solution 1:1000, Parke-Davis and Company, Detroit, Michigan.

TABLE 1. BLOOD PATTERN IN NORMAL DOG

Source	No. of dogs	RBC—millions per cu. mm.	WBC—thousands per cu. mm.	% of total WBC					
				Bas.	Eos.	Stabs.	Neut.	Lym.	Mono.
This lab.	50	6.82	11.9	0.2	6.1	2.5	62.5	27.0	1.7
Mayerson (1930)	60	6.49	11.2	0	2.0	0	74.0	20.0	4.0
Scarborough (1931)	700	7.20	11.8	0.7	5.0	0	68.2	20.0	6.1
Morris, *et al.* (1940)	35	6.20	11.5	0	5.4	0	71.8	21.8	1.0

indicated in the appropriate tables. Epinephrine was diluted with 100 ml. normal saline and administered intravenously as a continuous infusion, the time required for infusion being 45 to 60 minutes in all cases. During the epinephrine studies blood sugars were determined according to the method of Somogyi (1937). The dogs used in the epinephrine and thyroid administration studies were tested also for their responsiveness to ACTH. All dogs were fasted overnight prior to and during the tests.

Complete blood counts were made at the times and under the conditions indicated in the charts. Direct eosinophile counts were made by a modification of Dunger's method (1910).

TABLE 2. TYPICAL EFFECTS OF BILATERAL ADRENALECTOMY AND OF SIMPLE HYPOPHYSECTOMY ON THE CIRCULATING BLOOD ELEMENTS

Time postop.	RBC	WBC	Bas.	Eos.	Stabs.	Neut.	Lym.	Mono.
Bilateral adrenalectomy								
0	6.01	11,900	0	9.4	0.5	57.0	32.7	0.4
4 mo.	5.14	16,317	0	15.7	0.3	41.7	41.3	1.0
12 mo.	6.96	14,600	0.5	5.0	0.8	24.3	67.7	1.7
Simple hypophysectomy								
0	6.11	9,847	0	8.7	1.4	52.3	34.2	3.4
4 mo.	6.43	10,800	0	7.8	1.0	52.6	33.6	5.0
9 mo.	6.76	11,340	0	10.2	1.3	48.6	36.4	3.5
15 mo.	6.31	11,540	0.3	12.2	0.3	46.9	38.0	2.3

RESULTS

All counts on normal dogs were made in the morning in the post-absorptive state. The results obtained in this laboratory (Table 1) show a satisfactory agreement with those reported by Mayerson (1930), by Scarborough (1931), and by Morris *et al.* (1940).

The effects of bilateral adrenalectomy and of simple hypophysectomy on the circulating blood pattern were studied in detail in three dogs. The typical response in one dog of each group is recorded in table 2. Each count represents the average of numerous counts made at the postoperative period indicated. The adrenalectomized dogs were maintained in good

condition with implanted DOCA pellets.[3] The completeness of removal of the glandular hypophysis was checked by serial sections of the sellar contents made after autopsy. The effect of bilateral adrenalectomy on the cytology of the glandular hypophysis is shown in plate 1-C.

It is evident from the cell count of the altered glandular hypophysis recorded in the legend of plate 1-C that adrenal cortical hormone fractions other than those concerned with salt and water balance are necessary for the normal maturation of the hypophysial eosinophile cells.

The effects of pituitary hormone preparations on the circulating blood elements in three adrenalectomized and in three simply hypophysectomized

TABLE 3. EFFECTS OF EXOGENOUS HORMONE ADMINISTRATION TO DOGS AFTER BILATERAL ADRENALECTOMY AND AFTER SIMPLE HYPOPHYSECTOMY

Hormone preparation	Dosage and route of administration	Period of administration—days	Time after operation	Decrease*			Increase*		
				Eos.	Lym.	Neut.	Eos.	Lym.	Neut.
			Bilateral Adrenalectomy						
ACTH[1]	5 mg.—3× daily I.M.	13	4 mo.		0	0	+	0	0
Preloban Niphanoid	150 hypophysectomized rat units daily I.M.	3	5 mo.	0	+		0		++
Growth hormone[2]	0.5 mg./kilo daily S.C.	14	10 mo.	0	0	0	0	0	0
Thyrotropic hormone[3]	9 mg. daily S.C.	7	12 mo.	0	0		0	0	+
			Simple Hypophysectomy						
ACTH[1]	6 mg. I.V.	Single inj. pre & 4 hrs. post inj.	2 mo.	+++	++				+
	5 mg.—3× daily I.M.	6	8 mo.	+	+				+
Preloban Niphanoid	150 hypophysectomized rat units daily I.M.	3	9 mo.	++	++				++
Growth hormone[2]	0.5 mg./kilo daily S.C.	10	14 mo.	+	+				+
Desiccated pork thyroid daily	0.1 gm./kilo per os. daily	3	9 mo.	0	0	0	0	0	0

* 0—less than 10%; +—10 to 25%; ++—25 to 50%; +++—over 50%.
[1] Armour Lot No. 60–61.
[2] Armour Lot. No. 22KR1.
[3] Armour Lot No. 2D3.

dogs are shown in table 3. The results were similar in all the dogs in each group. They indicate that the hormones of the anterior pituitary do not influence the circulating blood elements directly but rather through the release of adrenal cortical hormones. After hypophysectomy atrophy of the inner two zones of the adrenal cortex gradually takes place. The stimulating effect of ACTH, of preloban and of growth hormone on the adrenal cortex is diminished after hypophysectomy. The degree of decreased effect increases with time after the hypophysectomy. Prolonged administration of hormones trophic to the adrenal cortex ultimately would be expected to restore the atrophic adrenals to such an extent that the amount of adrenal cortical hormone released on stimulation by an adrenotrophic

[3] The DOCA used was in the form of desoxycorticosterone acetate pellets. They were supplied by Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

TABLE 4. EFFECTS OF OVERACTION OF PITUITARY-ADRENAL CORTEX COMPLEX AS PRODUCED BY DENERVATION OF THE NEURAL HYPOPHYSIS*

Time postop.	RBC	WBC	Bas.	Eos.	Stabs.	Neut.	Lym.	Mono.
0	6.81	11,650	0	9.1	4.2	60.5	25.2	1.0
1 mo.	6.76	11,050	0	9.4	2.4	64.0	23.0	1.2
2 mo.	6.71	14,850	0	3.3	2.3	72.8	20.2	1.4
3 mo.	6.55	12,380	0	1.2	2.9	83.3	11.7	0.9
8 mo.	6.43	14,970	0	4.2	3.4	80.2	12.2	0
15 mo.	6.55	17,300	0	8.5	2.6	73.2	13.6	2.1
24 mo.	6.25	13,800	0	5.3	1.8	76.0	16.4	0.5
48 mo.	6.55	12,725	0	7.0	1.5	75.0	15.0	1.5

* Results represent averages of the counts made on 6 dogs.

hormone would again equal or approach that exhibited by the normal dog.

The effects on the circulating blood pattern of denervation of the neural hypophysis, leaving the glandular hypophysis in situ, are shown in table 4. The results indicate an overaction of the pituitary-adrenal hormone complex inasmuch as the numbers of circulating eosinophiles and lymphocytes are decreased.

TABLE 5. TYPICAL EFFECTS OF VARIOUS EXOGENOUS PITUITARY HORMONES ON THE CIRCULATING BLOOD ELEMENTS OF DOGS AFTER DENERVATION OF THE NEURAL HYPOPHYSIS

Hormone preparation	Dosage and route of administration	Period of administration days	Time after operation	Decrease*			Increase*		
				Eos.	Lym.	Neut.	Eos.	Lym.	Neut.
ACTH†	5 mg. subcutaneously	Single inj. counts pre & 4 hrs. post inj.	9 mo.	++	++				+
ACTH†	10 mg. subcutaneously	Single inj. counts pre & 4 hrs. post inj.	9 mo.	+++	++				++
ACTH†	15 mg. subcutaneously	Single inj. counts pre & 4 hrs. post inj.	9 mo.	+++	+++				++
Preloban niphanoid	150 hypophysectomized rat units daily i.m.	3	10 mo.	0	++		0		+
Whole post. pituitary extract	45 I.U. daily i.m.	30	9 mo.	0	0	0	0	0	0

* 0 = less than 10%; + = 10% to 25%; ++ = 25% to 50%; +++ = over 50%.
† Armour Lot No. 60-61.

The typical effects of various exogenous hormones on the circulating blood elements of six dogs with the neural hypophysis denervated are shown in table 5. It is evident that the adrenal cortex is preserved so that ACTH and preloban are still effective in initiating significant eosinophile and lymphocyte responses.

The effects on the circulating blood elements in the normal dog of the administration of ACTH, growth hormone* and preloban are shown in

* In experiments performed since this manuscript was submitted for publication it was found that a highly purified growth hormone preparation supplied by Armour & Company had no significant effects on the total white blood count, on the circulating

TABLE 6. EFFECTS OF ACTH, GROWTH HORMONE AND PRELOBAN ON THE CIRCULATING BLOOD ELEMENTS IN THE NORMAL DOG*

Hormone preparation	Dosage and route of administration	Hours after administration	% decrease			% increase		
			Eos.	Lym.	Neut.	Eos.	Lym.	Neut.
ACTH[1]	0.4 mg./kilo i.m.	4	64	24	—	—	—	13
ACTH[1]	0.4 mg./kilo i.v.	4	78	36	—	—	—	29
ACTH[1]	0.15 mg./kilo i.m.	4	—	11	0	12	—	0
ACTH[1]	0.15 mg./kilo i.v.	4	56	12	—	—	—	5
Growth hormone[2]	0.5 mg./kilo i.m.	4	24	5	0	—	—	0
Growth hormone[2]	0.5 mg./kilo i.v.	4	48	22	—	—	—	11
Preloban niphanoid	15 hypophysectomized rat units/kilo i.m.	4	23	12	—	—	—	6
Preloban niphanoid	45 hypophysectomized rat units/kilo i.m.	4	52	11	—	—	—	7

* These results represent the averages of observations on two dogs.
[1] Armour Lot No. 60–61.
[2] Armour Lot No. 22KR1.

TABLE 7. EFFECTS OF THYROIDECTOMY AND OF THYROIDECTOMY-GONADECTOMY ON CIRCULATING BLOOD ELEMENTS IN THE DOG*

Time after operation	RBC	WBC	Bas.	Eos.	Stabs.	Neut.	Lym.	Mono.
				Thyroidectomy				
0	6.77	11,050	0	4.7	2.5	55.3	34.9	2.6
1 mo.	6.58	12,675	0	6.0	2.0	57.4	32.5	2.1
3 mo.	6.04	12,950		4.5	1.5	63.0	29.0	2.0
6 mo.	6.39	13,650	0	3.5	2.5	59.0	30.5	4.5
				Thyroidectomy & Gonadectomy				
9 mo.	5.65	12,700		5.4	0.8	60.3	31.8	1.7
15 mo.	7.03	11,750	0	7.5	2.2	56.1	33.2	1.0

* Results are the average counts of two dogs in each group.

table 6. Of these three hormones, the first two are derived from the hypophysial eosinophile cells alone, while the last is an extract of the whole glandular hypophysis.

The effects of thyroidectomy and of thyroidectomy-gonadectomy on the circulating blood elements in two dogs of each type are shown in table 7. No significant effects were noted. The lymphoid tissue of the body and the thymus gland also were found to be normal at autopsy. Such dogs are quite sensitive to ACTH as regards its effect on the circulating blood elements.

Overaction of the pituitary-thyroid hormone complex was induced in

eosinophiles or lymphocytes, when administered in amounts comparable to those listed in the experiments tabulated in this paper. It is quite probable that the effects attributed to growth hormone in this paper may be due to a contamination of the preparation used with ACTH.

TABLE 8. TYPICAL EFFECTS OF VARIOUS EXOGENOUS HORMONES ON THE CIRCULATING BLOOD ELEMENTS IN THE NORMAL DOG

Hormone preparation	Dosage and route of administration	Period of Administration days	Decrease*			Increase*		
			Eos.	Lym.	Neut.	Eos.	Lym.	Neut.
Thyrotropic hormone[1]	9 mg. subcutaneously daily	6	0	+	0	0		0
	18 mg. subcutaneously daily	21	0	0	0	0	0	0
Desiccated pork thyroid plus thyrotropic hormone[1]	0.1 gm./kilo per os daily 9 mg. subcutaneously daily	8	0	0	0	0	0	0
Desiccated pork thyroid	0/1 gm./kilo per os daily	28	0	0	0	0	0	0
		58	0	0	0	0	0	0
		86			+	+	+	
Whole posterior pituitary extract	45 I.U. i.m. daily	45		+		+		+

* 0—less than 10%; +—10% to 25%; ++—25% to 50%; +++—over 50%.
[1] Armour Lot No. 2D3.

three dogs of each group by the administration of thyrotropic hormone, thyrotropic hormone plus desiccated thyroid substance, desiccated thyroid substance alone and whole posterior pituitary extract. That such measures fail to modify the circulating blood elements is evident from the results shown in table 8. In other experiments it was found that in two thyroidectomized dogs thyrotropin (Armour Lot #2D3), 9 mg. daily given for 14

TABLE 9. EFFECTS OF CORTISONE* ON THE CIRCULATING BLOOD ELEMENTS IN THE NORMAL DOG†

Dosage and route of administration	Period of administration days	RBC	WBC	EOS. per cu. mm.	% total WBC		
					Eos.	Lym.	Neut.
0	0	6.17	15,650	739	4.70	27.9	65.4
100 mg. daily i.m.	8	6.34	20,450	51	0.25	20.9	74.3
100 mg. daily i.m.	17‡	5.77	26,800	17	0.06	17.5	76.9
100 mg. daily i.m.	28‡	5.04	30,750	238	0.78	14.7	81.0

* Cortone-Merck, a saline suspension of 25 mg. cortisone per ml. of saline.
† These results represent the averages of four dogs. The per cent of eosinophiles was calculated from the direct eosinophile counts and the per cent of lymphocytes and neutrophiles by the differential counting method. All counts were made in the morning 24 hours after administration of the hormone.
‡ During these periods 4 to 6 nucleated red cells were observed per 400 white cells counted as contrasted with a negative finding in the normal untreated dog.

and for 22 days decreased the circulating eosinophiles more than 50 per cent and the lymphocytes up to 50 per cent. This effect is attributed to an 11 per cent contamination of the thyrotropic hormone with ACTH.[4] Apparently thyroidectomy sensitizes the body to ACTH because no such effect was obtained in the normal dog given the same thyrotropin.

The effects of cortisone on the circulating blood elements of the dog are

[4] Assay by Biochemical Research Division, Armour and Company, Chicago, Illinois.

TABLE 10. TYPICAL EFFECTS OF EXOGENOUS EPINEPHRINE ON THE CIRCULATING BLOOD ELEMENTS IN THE NORMAL DOG

Dog	Epinephrine administered	Hours after administration	WBC	Eos. per cu. mm.	% total WBC*		
					Eos.	Lym.	Neut.
B-8	0.05 mg.	0	11,150	590	5.3	28.0	59.5
		4.5	17,750	174	1.6	17.5	78.0
B-10	0.05 mg.	0	9,300	140	1.5	37.0	62.0
		4	17,200	26	0.2	24.5	74.0
B-12†	0.05 mg.	0	11,950	230	1.9	28.2	68.7
		4	12,750	182	1.4	24.0	71.2
B-12	0.18 mg.	0	14,650	346	2.3	30.0	62.5
		4	17,100	257	1.5	23.4	73.0
B-12	0.40 mg.	0	14,700	162	1.1	25.9	68.0
		4	19,250	34	0.2	18.6	74.2

* The per cent of eosinophiles was calculated from the direct eosinophile count and the per cent of lymphocytes and neutrophiles determined by the differential counting method.
† Note B-12 is less sensitive to the effect of exogenous epinephrine on the circulating eosinophiles.

presented in table 9. There resulted a slight decrease in the number of circulating red cells, and marked decreases in the circulating eosinophiles and lymphocytes. The eosinophile decrease was not so well maintained as was that of the lymphocytes. The neutrophiles were increased definitely.

TABLE 11. INFLUENCE OF HYPOPHYSECTOMY, OF ADRENALECTOMY AND OF THYROIDECTOMY-GONADECTOMY ON THE EFFECTS OF EXOGENOUS EPINEPHRINE ON THE CIRCULATING BLOOD ELEMENTS OF THE DOG

Dog	Hormone administered	Hours after administration	WBC	Eos. per cu. mm.	% total WBC*		
					Eos.	Lym.	Neut.
B-10	Epinephrine	0	9,300	140	1.5	36.0	62.0
Normal	0.05 mg.	4	17,200	26	0.2	24.5	74.0
B-10	Epinephrine	0	10,600	133	1.2	37.0	62.5
Hypox 3 wks.	0.05 mg.	4	15,400	218	1.4	28.9	70.0
B-10	Epinephrine	0	11,750	471	4.0	46.5	50.5
Hypox 4 wks.	0.30 mg.	4	16,450	639	3.8	37.0	55.5
B-10	ACTH†	0	16,950	729	4.3	39.0	56.5
Hypox 4 wks.	6.0 mg. i.v.	4	16,850	182	1.1	27.0	72.2
K-41	Epinephrine	0	21,500	2670	12.4	47.8	37.5
Adrenal. 12 mo.	0.05 mg.	4	19,600	2060	10.5	38.5	48.1
TG-2	Epinephrine	0	14,300	1546	10.8	23.0	64.0
Thyroid.-Gonad. 9 mo.	0.05 mg.	4	14,550	373	2.6	20.0	74.5

* The per cent of eosinophiles was calculated from the direct eosinophile count and the per cent of lymphocytes and of neutrophiles determined by the differential counting method.
† Armour Lot No. 60–61.

At autopsy the lymphoid tissue was decreased generally and the thymus gland and spleen were small.

The effects of epinephrine on the circulating blood elements in twelve normal dogs (table 10) were found to be similar to those reported previously for the rat and for man (Recant *et al.*, 1950), and for the dog (Bertelli, Falta and Schweeger, 1910). A marked difference in sensitivity of normal dogs to exogenous epinephrine was noted. As indicated in the table,

TABLE 12. THE INFLUENCE OF DENERVATION OF THE NEURAL HYPOPHYSIS ON THE EFFECTS OF EPINEPHRINE ON THE CIRCULATING BLOOD ELEMENTS OF THE DOG

Dog	Hormone administered	Hours after administration	WBC	Eos. per cu. mm.	% total WBC*			Blood sugar response†
					Eos.	Lym.	Neut.	
101X. Operated 10 mo. Excellent Diabetes Insipidus	Epinephrine 0.3 mg.	0	16,550	439	2.6	31.5	64.5	
		4	20,100	393	1.9	27.2	68.5	42
101X. Operated 10 mo. Excellent Diabetes Insipidus	Epinephrine 0.5 mg.	0	18,800	409	2.1	18.6	78.0	
		4	19,000	289	1.5	18.2	77.5	39
101X. Operated 10 mo. Excellent Diabetes Insipidus	Epinephrine 0.9 mg.	0	13,800	479	3.5	17.0	76.0	
		4	17,200	512	3.0	11.5	81.0	85
101X. Operated 10 mo. Excellent Diabetes Insipidus	ACTH‡ 12 mg. i.v.	0	15,700	558	5.3	21.4	73.0	
		4	17,500	182	1.1	19.0	76.3	
104X. Operated 1 mo. Excellent Diabetes Insipidus	Epinephrine 0.4 mg.	0	22,200	2451	11.0	25.0	61.5	
		4	25,750	2973	11.5	30.5	56.0	35
105X. Operated 6 wks. Excellent Diabetes Insipidus	Epinephrine 0.4 mg.	0	15,500	273	1.8	34.7	60.7	
		4	14,850	490	3.3	37.2	57.8	48
103X. Same operation as above but no Diabetes Insipidus	Epinephrine 0.3 mg.	0	9,300	400	4.3	22.8	70.5	
		4	12,200	79	0.7	16.7	79.3	51

* Per cent eosinophiles were calculated from the direct eosinophile counts, the per cent lymphocytes and neutrophiles were determined by the differential counting method.

† This figure represents the increase in mg. per cent of blood sugar elicited by the epinephrine infusion. Bloods were drawn immediately before and 15–20 minutes after completion of the infusion.

‡ Armour Lot No. 60–61.

two of the dogs gave a good response to 0.05 mg. while another failed to give an adequate response to a much higher dosage. As shown in table 11 these effects are not eliminated in the dog subjected to both thyroidectomy and gonadectomy. They are eliminated by complete denervation of the neural hypophysis (table 12), by simple hypophysectomy and by bilateral adrenalectomy (table 11). The effect of epinephrine on the blood sugar level is unchanged by any of these procedures. It is evident that the failure

TABLE 13. THE INFLUENCE OF EXOGENOUS THYROID SUBSTANCE ON THE EFFECTS OF EPINEPHRINE ON THE CIRCULATING BLOOD ELEMENTS IN THE DOG

Dog	Dosage and duration of thyroid administration	Hormone administered	Hours after hormone administration	WBC	Eos. per cu. mm.	% Total WBC*			Blood sugar response†
						Eos.	Lym.	Neut.	
B-15	0	Epinephrine	0	9,300	2160	23.2	25.0	50.6	
		0.2 mg.	4	12,700	1023	8.0	19.0	69.2	35
B-15	0.2 gm./kilo per os daily.	Epinephrine	0	10,400	715	6.8	26.0	65.4	
	12 days	0.2 mg.	4	14,150	632	4.4	18.0	74.0	42
B-15	0.2 gm./kilo per os daily.	ACTH‡	0	12,200	1412	11.5	23.8	62.0	
	16 days	10 mg. i.v.	4	12,700	426	3.3	15.3	80.0	
B-15	0.2 gm./kilo per os daily.	Epinephrine	0	9,750	1457	14.7	26.0	58.2	
	24 days	0.3 mg.	4	13,000	1089	8.3	23.8	64.6	40
B-15	18 days after cessation of	Epinephrine	0	13,000	1432	11.0	20.4	67.3	
	thyroid administration	0.3 mg.	4	14,150	1212	8.6	18.4	71.0	57
B-15	32 days after cessation of	Epinephrine	0	9,750	1343	13.8	23.5	61.0	
	thyroid administration	0.3 mg.	4	12,200	423	3.5	17.1	75.6	46
B-15	50 days after cessation of	Epinephrine	0	12,600	853	6.8	24.6	64.9	
	thyroid administration	0.3 mg.	4	13,550	296	2.2	15.4	78.6	51
B-16	0	Epinephrine	0	9,400	529	5.6	22.5	70.5	
		0.4 mg.	4	12,000	267	2.2	17.0	78.4	31
B-16	0.2 gm./kilo per os daily.	Epinephrine	0	12,300	281	2.3	20.5	75.5	
	12 days	0.4 mg.	4	14,500	481	3.3	17.5	77.3	29
B-16	0.2 gm./kilo per os daily.	ACTH‡	0	17,100	790	4.6	17.0	75.5	
	15 days	7 mg. i.v.	4	20,350	212	1.1	12.0	84.0	
B-16	0.2 gm./kilo per os daily.	Epinephrine	0	11,100	607	5.4	17.2	75.0	
	24 days	0.4 mg.	4	12,250	510	4.1	18.2	74.0	36
B-16	20 days after cessation of	Epinephrine	0	10,150	225	2.2	22.4	73.5	
	thyroid administration	0.4 mg.	4	16,250	731	4.5	19.0	74.0	42
B-16	40 days after cessation of	Epinephrine	0	14,250	378	2.7	13.1	80.5	
	thyroid administration	0.4 mg.	4	16,100	811	5.0	13.0	79.0	58
B-16	60 days after cessation of	Epinephrine	0	10,300	696	6.7	13.2	76.3	
	thyroid administration	0.4 mg.	4	11,100	570	5.1	10.9	80.5	63
B-16	65 days after cessation of	ACTH‡	0	7,650	1040	13.6	12.1	71.5	
	thyroid administration	7.0 mg. i.v.	4	12,350	575	4.6	8.4	83.4	
B-16	90 days after cessation of	Epinephrine	0	9,800	1954	19.9	10.7	68.8	
	thyroid administration	0.4 mg.	4	10,750	1251	11.6	7.5	78.0	53
B-16	5 mo. after cessation of	Epinephrine	0	8,800	500	5.7	14.5	77.3	
	thyroid administration	0.4 mg.	4	10,500	250	2.4	10.6	82.3	48

* Per cent eosinophiles were calculated from the direct eosinophile counts, the per cent lymphocytes and neutrophiles were determined by the differential counting method.

† This figure represents the increase in mg. per cent of blood sugar elicited by the ephinephrine infusion. Bloods were drawn immediately before and 15–20 minutes after completion of the infusion.

‡ Armour Lot No. 60–61.

Dog B-16 developed a marked exophthalamus which became noticeable about the tenth day of thyroid administration and persisted for approximately 3 months after cessation of thyroid.

of epinephrine to modify the circulating blood elements of recently hypophysectomized dogs and of dogs with the neural hypophysis denervated is not due to a functional inability of the adrenals as such dogs respond readily to the administration of ACTH (tables 11 and 12).

It has been our finding that approximately one normal dog in four fails to show a drop in circulating eosinophiles and lymphocytes when epinephrine is administered in dosages adequate to cause a maximum response in other dogs. Also it has been observed repeatedly that dogs whose circulating blood elements respond typically to an initial dose of epinephrine,

frequently will show little response to a second dose, even though large, which is given several hours after the first dose.

In table 13 are shown the effects of epinephrine on dogs given thyroid gland substance for prolonged periods. These dogs had all shown a normal response to epinephrine prior to the administration of the thyroid gland substance. After the administration of the thyroid with a dosage and a time period of administration such as to cause obvious and marked excitation of the nervous system the dogs failed to respond to epinephrine with changes in the circulating blood elements. The return to a normal response following cessation of thyroid administration was gradual requiring weeks to months.

ANALYSIS OF RESULTS

On analysis the results indicate that in the dog the pituitary-adrenal hormone complex exerts a modifying influence on the number of circulating blood elements, on the bone marrow, and on the thymic and lymphoid tissue of the body. Overactivity of this complex results in a decrease in circulating eosinophiles and lymphocytes and a concomitant increase in circulating neutrophiles. The thymic and lymphoid tissues of the body are also reduced. Underaction of the pituitary-adrenal hormone complex has opposite effects. Slight anemia may result and the circulating eosinophiles and lymphocytes are increased. The thymic and the lymphoid tissues of the body are increased.

The pituitary-thyroid hormone complex has no such specific effects on the circulating blood elements, or on the thymic and lymphoid tissues. Depression of circulating eosinophiles and lymphocytes usually is not obtained in the normal dog on the administration of so-called purified thyrotropic hormone or of dried thyroid gland substance. When such a depression of eosinophiles and lymphocytes occurs in the thyroidectomized dog given commercial thyrotropic hormone the effects as before stated are ascribed to an 11 per cent contamination of the hormone with ACTH as well as sensitivity of the thyroidectomized dog to ACTH. This explanation is attested to by the failure of the thyrotropic hormone to have such an effect in either the adrenalectomized dog or the normal dog (tables 3 and 8).

The pituitary hormones which have modified the circulating blood elements in our dogs are ACTH and growth hormone. Of these ACTH presumably is the more significant. Its administration in the dog causes effects similar to those obtained after complete denervation of the neural hypophysis (tables 4 and 6). In dogs so modified the glandular hypophysis is characterized by an increase in the number of eosinophiles and a marked decrease or absence of basophile cells (plate 1-D). In such dogs the zona

fasciculata of the adrenal cortex hypertrophies (plate 1-E) and the thyroid gland atrophies. After an interval of three or more years, the basophile cells reappear but their function is depressed (Heinbecker and Pfeiffenberger, 1950, loc. cit.). They are hyalinized or degranulated (plate 1-F). The changes in them are similar to those described by Crooke (1935) as being found characteristically in Cushing's syndrome in man. In such dogs with a denervated neural hypophysis insulin sensitivity is decreased, the number of circulating red cells is normal or increased, and the circulating eosinophiles and lymphocytes are decreased. The thymus and the lymphoid tissues are reduced markedly (Heinbecker and Pfeiffenberger, 1950, loc. cit.). Despite the appearance of the altered basophile cells, three or more years following denervation of the neural hypophysis, the adrenal cortex remains hypertrophied and the thyroid gland atrophic (plate 1-G). The alterations in the circulating blood elements and in the thymus and the body lymphoid tissue remain unchanged also.

In man a similar decrease in circulating eosinophiles and lymphocytes together with a decrease in thymic and lymphoid tissues has been found associated with hypophysial eosinophile cell overaction as evidenced by hypertrophy of the adrenal glands. Such changes occur in Cushing's syndrome due to a primary atrophy of the paired paraventricular nuclei (Heinbecker and Pfeiffenberger, 1950, loc. cit.). The alterations in the circulating blood elements indicate that the hypertrophied adrenals release an excess of those adrenal corticoids which modify the circulating blood elements. The hypertrophy of the adrenals is attributed to the trophic influence of the excess ACTH released by the overactive hypophysial eosinophile cells. After the exogenous administration of cortisone in dogs the zona fasciculata of the adrenal cortex atrophies (unpublished data obtained in this laboratory and by others). It is concluded that the zona fasciculata is the principal source of the adrenal cortical hormone fractions, the C 11–17 oxysteroids, which modify the circulating blood elements and the thymic and lymphoid tissues. Among the workers responsible for establishing that the C 11–17 oxysteroids of the adrenal cortex are responsible for modifying the circulating blood elements, the thymus and the lymphoid tissue elements are Sayers and Sayers (1949) and Recant, Hume, Forsham and Thorn, loc. cit. (1950).

Evidence as to the manner in which the number of circulating eosinophiles and lymphocytes is modified by these corticosteroids has been offered by Dougherty and White, loc. cit. (1944) and by others. The results of our studies are in agreement with their evidence that excess adrenal corticosteroids act by destroying these circulating blood elements while they are inhibiting their production for release into the circulation. A decrease in the adrenal corticosteroids is associated with an augmentation of

eosinophile and lymphocyte production and a decrease in their destruction in the circulating blood.

The factors which modify the release of ACTH are the activity of the hypophysial cells and the level of the corticosteroids in the circulating blood. An increase in the number and activity of the hypophysial eosinophile cells has been shown by us to occur in the dog after denervation of the neural hypophysis. Sayers and Sayers (1947) have presented evidence that one factor in controlling ACTH release is the amount of the cortical hormones in circulation. Their conclusion is supported by our evidence that the zona fasciculata of the adrenal cortex atrophies after exogenous cortisone administration.

Another mechanism for the release of ACTH is the action of epinephrine. This follows from the evidence that after the exogenous administration of epinephrine the circulating blood elements are modified in a manner similar to that which follows the administration of ACTH (Recant, Hume, Forsham and Thorn, loc. cit., 1950) and others. The manner in which epinephrine effects the release of ACTH has been difficult to determine. On the basis of our experimental work, epinephrine when it results in a release of ACTH is considered to initiate this effect by stimulating the cerebral cortex. The activity in turn results in a depression of those hypothalamic nuclei which innervate and stimulate the secretory activity of the neural hypophysis. A decrease in the effective secretion of the neural hypophysis, particularly the fraction released by the fibers from the paired paraventricular nuclei, results in an endocrine imbalance through which the eosinophile cells of the glandular hypophysis are stimulated to secrete ACTH. The evidence that epinephrine fails to effect ACTH release after denervation of the neural hypophysis indicates that epinephrine acts through the nervous system. In dogs with the neural hypophysis completely denervated (table 5) the C 11–17 oxysteroids still are released from the adrenal gland in normal amounts after ACTH stimulation. Epinephrine still effects a normal blood sugar response in such dogs. The interpretation offered is that because the hypothalamico-hypophysial system is interrupted excitation of the cerebral cortex by epinephrine can no longer depress the posterior lobe hormone secretion. Such depression effected by denervation of the neural hypophysis has been shown to increase the activity of the eosinophile cells of the glandular hypophysis in the dog (Heinbecker, 1944).

McDermott, Fry, Brobeck and Long (1950) have presented evidence which supports the concept that one of the mechanisms for the release of adrenocorticotrophic hormone by epinephrine is a direct stimulation of the anterior lobe of the pituitary. After the instillation of epinephrine directly into the anterior chamber of the eye containing an engrafted anterior

pituitary they obtained a fall in circulating eosinophiles. Injection of the same amount of epinephrine into the opposite or normal eye produced no significant fall in the eosinophile count. After the surgical removal of the eye bearing the graft subcutaneously injected epinephrine produced no effect on the circulating eosinophiles. One must conclude that under the conditions of their experiment epinephrine stimulated directly the cells of the glandular hypophysis to release ACTH. However, the conditions of their experiment were different from ours. In our dogs with the neural hypophysis denervated but with the glandular hypophysis left *in situ* a dose of epinephrine normally effective when acting on the glandular hypophysis through the blood stream did not alter the circulating blood elements. Apparently the concentration of epinephrine within the transplanted hypophysis in their experiments was higher than in ours, higher than could be applied to the hypophysis in situ through the blood stream without toxicity to the animal.

Pertinent to this problem is the observation that epinephrine injected directly into the anterior lobe of the rabbit (Markee, Sawyer and Hollinshead, 1948) has been shown to release the luteinizing hormone which, like ACTH, is derived from the hypophysial eosinophile cells. Injected through the blood stream, amounts of epinephrine many times greater than the amount injected directly into the anterior lobe are not adequate to effect a similar release of luteinizing hormone (Markee, Sawyer and Hollinshead, 1948). Denervation of the adrenal glands does not prevent the ovulation associated with coitus in the rabbit (Brooks, 1938). Severance of the hypothalamico-hypophysial tract, on the other hand, prevents the ovulation associated with coitus in the rabbit (Brooks, 1938, loc. cit.). The release of epinephrine into the blood stream in physiological dosages cannot be regarded as the mechanism through which ovulation is affected. A neurogenic mechanism is postulated by Brooks (1937).

The effect of intravenously administered and of locally applied epinephrine on the central nervous system has been studied in curarized cats by S. H. Bartley and Peter Heinbecker in this laboratory (fig. 3), by simultaneously recording the electrical activity of the exposed sensori-motor cortex and of the hypothalamic region using the cathode-ray oscillograph as a recording mechanism. In these studies it was observed that with low dosages of intravenously injected epinephrine excitation was seen in the cortex along with depression of activity in the hypothalamus. More recently O'Leary and his associates (personal communication) have observed that when the dosage of epinephrine is increased sufficiently electrical activity in the hypothalamus is increased as well as in the cortex. Excitation of the activity of the central nervous system by epinephrine has been reported also by Grinker and Serota (1938). If of sufficient degree such

activity could prevent inhibitory pathways from the cortex from effecting the depression of the hypothalamus essential for the release of ACTH.

To test further the concept that epinephrine causes the release of ACTH by depression of the hypothalamic nuclei innervating the neurohypophysis, dried thyroid substance was administered to dogs which previously were shown to respond normally to exogenous epinephrine administration as far as modification of their circulating blood elements was concerned. After the thyroid substance had excited the dogs obviously and markedly, epinephrine failed to cause the release of ACTH (table 13) as indicated by a failure to cause a depression in the number of circulating eosinophiles and lymphocytes. The explanation offered is that the inhibitory mechanisms from the cortex to the hypothalamus then were no longer effective because of the generalized excitation of the central nervous system. Epinephrine fastness within the central nervous system could be explained similarly as due to a failure of inhibitory mechanisms at a time when excitation throughout the nervous system exists as a consequence of a first dose.

The elimination of the effect of epinephrine on the circulating blood elements by an excess of thyroid hormone affords an explanation for the adrenal cortical deficiency in the hyperthyroidism of man. In such hyperthyroidism generalized cerebral excitation would be expected to dominate over the inhibitory mechanisms which might normally act on the hypothalamico-hypophysial system to depress the hypothalamic nuclei innervating the neural hypophysis. Such depression is deemed necessary for the normal functioning of the pituitary-adrenal hormone mechanism. Augmented excitation of the hypothalamic nuclei which innervate the neural hypophysis would be expected to cause an increase in the secretion of the neural hypophysis. This condition has been simulated by the administration of an excess of exogenous whole posterior pituitary extract. It resulted in an increase of hypophysial basophile cells which are trophic to the thyroid gland (Heinbecker, 1949) (plate 1-H). With prolonged excitation of the hypothalamic nuclei and increased secretion of the neural hypophysis as in persons with hyperthyroidism, it would be expected that adrenal cortical hypofunction would occur. With it there would result an increase in hypophysial basophile cells and a decrease in hypophysial eosinophile cells. The inner two zones of the adrenal cortex would be expected to atrophy and with such atrophy a depression of their function would be anticipated. This would increase the circulating lymphocytes, the body lymphoid tissue and cause an enlargement of the thymus as shown by the effect of adrenalectomy in the dog. The glandular hypophysis in man with hyperthyroidism shows an increase in basophile cells and a decrease in eosinophile cells (Plate 1-I). With such changes there occurs

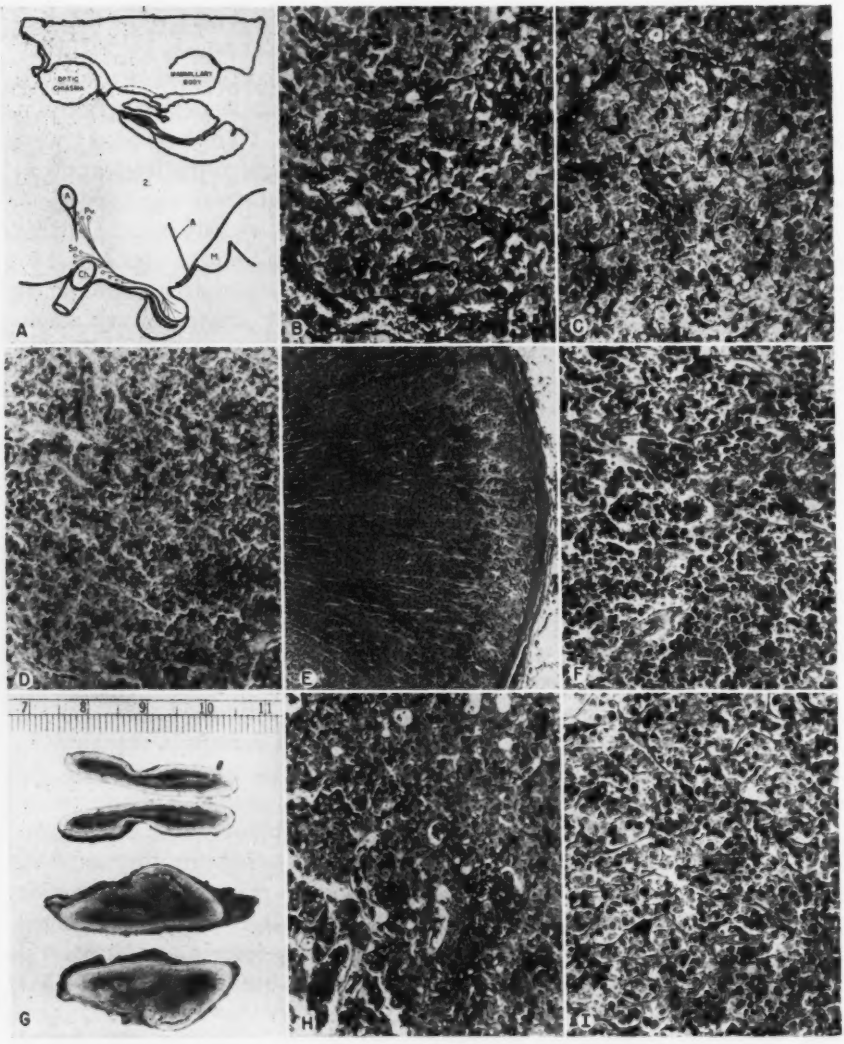

PLATE 1

A: Diagrams indicating lesions made: (1) To denervate completely the neural hypophysis. (2) To interrupt afferent pathways excitatory to the paired paraventricular nuclei. A indicates site of lesion.

B: Photomicrograph (×130) of the glandular hypophysis of a normal dog. Cell count:*

* All pituitaries are serially sectioned. Counts are made on representative sections according to the method of A. T. Rasmussen.

frequently an increase in circulating lymphocytes, an increase in body lymphoid tissue, and an enlargement of the thymus gland.

The interpretation of the mechanism of action of epinephrine and of thyroid gland substance on the nervous system is based on experimental evidence obtained on the dog (Heinbecker, White and Rolf, 1947). Thus, in the dog it has been shown that two hypothalamic nuclei alone contribute to the innervation of the neural hypophysis. These are the paired supraoptic and the paired paraventricular nuclei. On denervation of the neural hypophysis all of the cells of the supraoptic nuclei and about 50 per cent of the rostral and ventral cells of the paired paraventricular nuclei undergo retrograde degeneration. Similar findings have been reported by Rasmussen (1940). It has been shown also that when a lesion is made rostral to the mammillary bodies (plate 1-A) the cells of the paired paraventricular nuclei do not undergo retrograde degeneration. When a lesion is made so that the paired paraventricular nuclei are transected the cells of the nuclei caudal to the section undergo retrograde degeneration but those rostral to the lesion do not. Similar changes in the cytology of the glandular hypophysis, i.e. eosinophile cell preponderance with basophile cell loss, result from both a lesion rostral to the mammillary bodies and when the entire neural hypophysis is denervated. Bodily changes, except for diabetes insipidus, such as decreased insulin sensitivity, obesity, hypertension, enlargement of the adrenal glands, etc., are similar for both groups of dogs. The interpretation offered is that

Chrom.—52.3%, Eos.—38.5%, Bas.—9.2%.

C: Photomicrograph (×130) of the glandular hypophysis of a dog in which one adrenal was removed 19 months previously and the remaining gland gradually destroyed by wrapping in silk. Cell count: Chrom.—70.8%, Eos.—21.2%, Bas.—8.0%.

D: Photomicrograph (×90) of the glandular hypophysis of a dog in which the neural hypophysis was denervated 25 months previously. Cell count: Chrom.—35.8%, Eos.—64.2%, Bas.—0%.

E: Photomicrograph (×90) of the adrenal cortex of a dog in which the neural hypophysis was denervated 25 months previously. The glandular hypophysis of this dog is illustrated in D.

F: Photomicrograph (×90) of the glandular hypophysis of a dog in which the neural hypophysis was denervated 96 months previously. Cell count: Chrom.—36.4%, Eos.—24.0%, Bas.—39.6%.

G: Adrenal glands of a dog in which the neural hypophysis was denervated 96 months previously as compared to those of a normal dog of comparable age and size. The glandular hypophysis of this dog is illustrated in F.

H: Photomicrograph (×90) of the glandular hypophysis of a dog given posterior lobe hormone, 25 units daily for 28 days. Cell count: Chrom.—30.9%, Eos.—51.9%, Bas.—17.2%.

I: Photomicrograph (×130) of the glandular hypophysis of a human male with hyperthyroidism, basal metabolic rate +62. Cell count: Chrom.—60.0%, Eos.—25.0%, Bas.—15.0%.

in the second group of dogs nerve fibers are interrupted which normally are excitatory to the paraventricular nuclei. When cortisone is administered in excess a marked polyuria develops and changes occur in the glandular hypophysis similar to those which occur after complete denervation of the neural hypophysis (unpublished data in this laboratory) and on the interruption of fibers excitatory to the paraventricular nuclei.

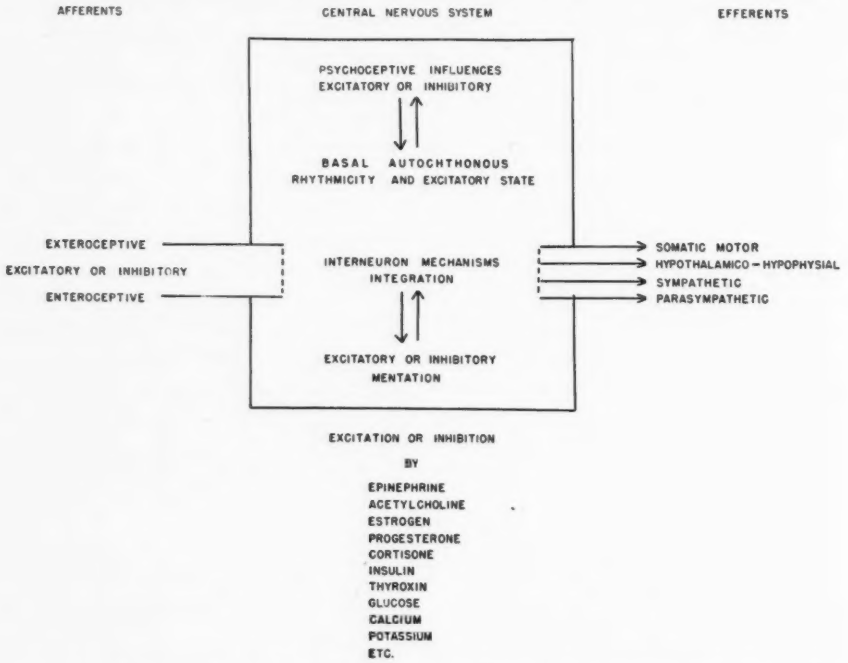

FIG. 1. Schema of organization of nervous system. Basic activity within the central nervous system is augmented or decreased by externally and internally aroused nervous influences. Humoral and chemical influences primarily affect activity within the central nervous system. Four efferent pathways are depicted.

Apparently fibers from the paraventricular nuclei are trophic to cells in the neural hypophysis which secrete a hormone which is in balanced relationship with hormones in the adrenal cortex, one of which is cortisone.

The organization and function of the nervous system is schematically depicted in fig. 1. The manner in which neuro-endocrine and endocrine interrelations are effected is shown schematically in fig. 2. The correctness of the concepts suggested is substantiated fully in the demonstration that all the signs and symptoms of Cushing's syndrome may result either from a lesion primary in the paraventricular nuclei of the hypothalamus or

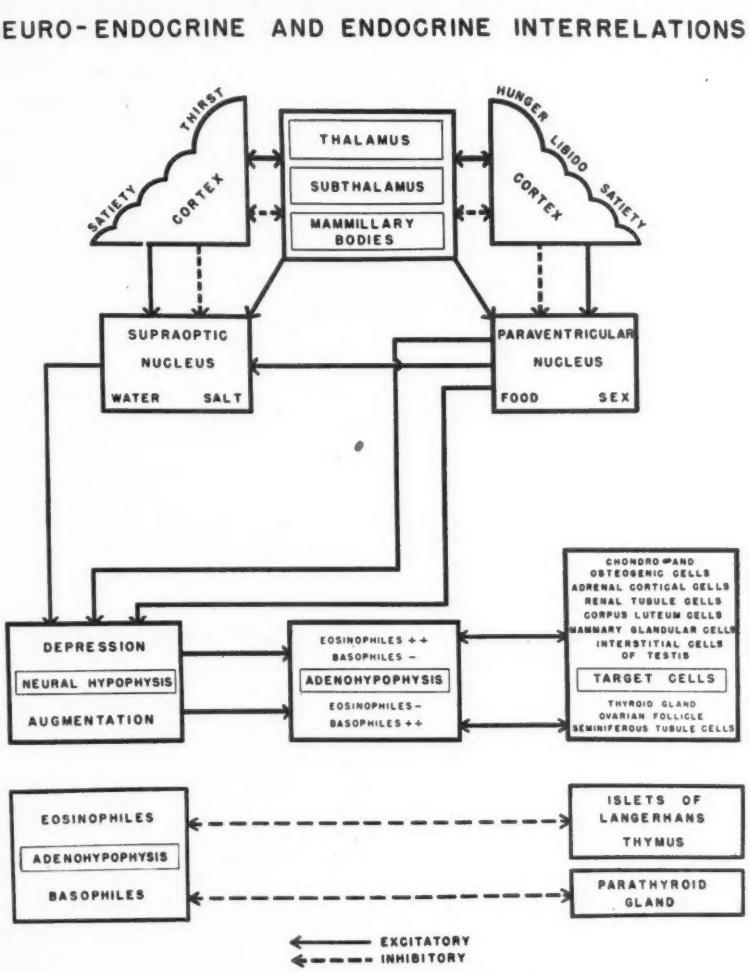

FIG. 2. Schema of neuro-endocrine and endocrine interrelations. The neural influences are exerted over the hypothalamico-hypophysial tract which innervates the neural hypophysis.

from a secretory tumor of the adrenal cortex. The chief manifestations of Cushing's syndrome have been reproducible in the dog by partial or complete denervation of the neural hypophysis. Changes in the glandular hypophysis identical with those found in human cases of Cushing's syndrome have been produced.

Having established the existence of an anatomical basis for modification of the glandular hypophysis by the central nervous system it is suggested

that functional influences, as they have been shown to act in the release of ACTH, may initiate endocrine disturbances similar to those which can be initiated by lesions primary in any endocrine gland. Constitutional susceptibility to influences of the nervous system on the glandular hypophysis and of the glandular hypophysis on the other endocrine glands may be dominant factors in determining which of the common endocrine disorders will be manifested by any particular individual.

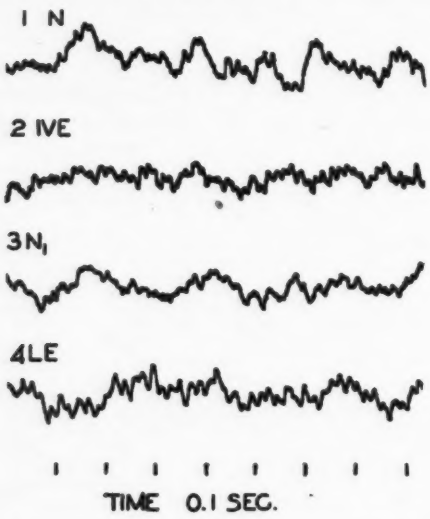

FIG. 3. Records of potential activity of sensori-motor cortex of cat as influenced by intravenously administered and locally applied epinephrine.

1N. normal control

2IVE. shows effect of 3 drops epinephrine 1:1000 administered intravenously. Note increased frequency of rhythm.

$3N_1$. Normal control

4LE. record after epinephrine applied (1:5000) on cotton placed on the surface of the sensori-motor cortex near the recording electrodes. Note increase in amplitude and frequency of activity pattern.

SUMMARY

The effects on the circulating blood elements and on lymphoid tissue by alterations in neuro-endocrine and endocrine interrelations of long duration have been studied in the dog.

Overaction of the hypophysial eosinophile-adrenal zona fasciculata endocrine complex results in a decrease in the number of circulating lymphocytes and eosinophiles and an increase in the number of neutrophiles. The threshold for depression of the eosinophiles is lower than that for depression of the lymphocytes but it is not maintained as well for long

periods. The lymphoid tissue of the thymus gland, the spleen and the lymph nodes is decreased.

Underaction of the hypophysial eosinophile-adrenal zona fasciculata endocrine complex permits an increase in circulating lymphocytes and eosinophiles and a decrease in the number of neutrophiles. The thymus and lymphoid tissue of the body generally are increased.

Overaction of the hypophysial eosinophile-adrenal fasciculata endocrine complex may be brought about experimentally by complete denervation of the neural hypophysis or clinically by a tumor of the adrenal cortex secreting an excess of a hormone fraction normally secreted by the zona fasciculata. Underaction of the hypophysial eosinophile-adrenal zona fasciculata endocrine complex may be brought about by removal of the pars distalis of the glandular hypophysis, by adrenalectomy, by overaction of the neural hypophysis, and by an excess of exogenously administered adrenal cortical hormone.

In normal dogs a decrease in the number of circulating lymphocytes and eosinophiles and an increase in the number of neutrophiles may be produced by the administration of exogenous adrenocorticotropic hormone, by growth hormone, by preloban, by cortisone and by epinephrine. The effects, except in the case of cortisone, are abolished in bilaterally adrenalectomized dogs.

Thyroidectomy, gonadectomy, and the administration of thyrotropic hormone, desiccated thyroid gland substance and posterior pituitary hormone to normal dogs have been found to be without appreciable effect on the circulating blood elements.

The release of ACTH by epinephrine is abolished in simply hypophysectomized dogs, in dogs with the neural hypophysis *completely* denervated, and in otherwise normal dogs with an excess of circulating thyroid hormone or of epinephrine. The evidence warrants the interpretation that epinephrine causes the release of ACTH by stimulating the cerebral cortex. Fibers from the cortex in turn depress the paired paraventricular nuclei of the hypothalamus. When the nuclei are depressed their efferent neurons do not activate the secretory cells of the neural hypophysis. A decrease in a factor in the neural hypophysial secretion results in increased maturation and overaction of the hypophysial eosinophile cells which secrete among other hormone fractions ACTH. If the general level of excitation within the central nervous system is too high to permit inhibition of the hypothalamic nuclei by influences from the cerebral cortex or if the cells of the paraventricular nuclei are themselves already excited above a certain level, epinephrine loses its capacity to inhibit the activity of the paraventricular nuclei and thus its capacity for ACTH release.

The sympathetic nervous system releases epinephrine by stimulation of the adrenal medulla. By stimulation of the cerebral cortex epinephrine

serves as the emergency mechanism for the release of ACTH via the hypothalamico-hypophysial system. Other slower acting but more constant nervous mechanisms exist which also act through the hypothalamico-hypophysial system to control the release of ACTH.

The increase in circulating lymphocytes, of lymphocytes in other tissues and the enlargement of the thymus observed in humans with hyperthyroidism is indicative of a decrease in adrenal cortical function. This decrease in function is attributed to a failure of inhibitory mechanisms within the central nervous system which normally are capable of depressing the paraventricular hypothalamic nuclei.

A concept of the mechanism of neuro-endocrine and endocrine interrelations is presented.

We wish to acknowledge the technical assistance of Miss Rosemary French in this work.

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SEASONAL RESPONSE OF FOLLICLES IN THE OVARIES OF THE BAT *MYOTIS GRISESCENS* TO PREGNANCY URINE GONADOTROPHIN

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INTRODUCTION

THE ovarian response to gonadotrophin depends upon the state of the ovary as well as upon the nature of the hormone and its method of administration. Previous studies (Guthrie and Jeffers, 1938) indicated that bats of the genus Myotis were useful for ovarian studies. Since bats have an annual estrous cycle and mature but a single ovum each year, factors conditioning follicular growth can be more easily distinguished than in polyestrous animals in which overlapping of phases occurs in the ovaries. Cytological studies of ovaries of normal bats throughout the estrous cycle have demonstrated variations in the total number of follicles and, of more significance, variations in the volume of the follicle-oocyte system (Guthrie, Jeffers, and Smith, 1951). The present paper considers the responsiveness of the ovary to PU gonadotrophin at various stages of the estrous cycle.

MATERIAL AND METHODS

The ovaries of 60 bats of the species *Myotis grisescens* collected at intervals from June through November were studied. The animals were injected with pregnancy urine gonadotrophin[1] for three or five days at twelve hour intervals for the autumn bats and eight hour intervals for the summer collections. At each treatment 0.1 of the dilution calculated to give the desired dosage for the particular experiment was injected subcutaneously.

The bats were killed by decapitation; the ovaries were fixed in Champy's fluid. Serial sections of both ovaries of each bat were cut at 4 microns and stained with acid fuchsin according to Kull. The size of each follicle was recorded as the two diameters at right angles through the largest section of the follicle. Two diameters of each oocyte were also recorded, not necessarily in the same section as the follicular diameters, since the oocyte is eccentrically located in the larger follicles. Measurements were made at a magnification of 600× using a screw micrometer one unit of which equalled 0.3 microns. A frequency distribution of the normal follicles classified according to the average diameter with a class interval of 3 microns was made for each ovary. The protoplasmic volume of the follicle-oocyte system was calculated using the average diameter. In the few vesicular follicles the antrum was measured as accurately as possible and its volume subtracted.

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[1] Parke-Davis' Antuitrin S.

OBSERVATIONS

Follicular growth: Follicular differentiation in the ovaries of newborn, immature, and mature females treated with pregnancy urine gonadotrophin is like that observed in untreated individuals (Guthrie, Jeffers, and Smith, 1951). In general the ovaries are slightly larger and the size of the largest follicle is increased in the treated bats as compared with the controls. Luteinization is observed only in the corpora lutea found after ovulations are induced.

TABLE 1. SEASONAL VARIATION IN FOLLICLES PER BAT AFTER PU INJECTION

Stage of estrous cycle	Collection	Total dosage R.U.	Number of individuals	Average number of follicles	Volume of follicle-oocyte system $\mu^3 \times 10^6$	Follicles with antra	Diameter of largest follicle μ
	6/18[a]	28.0	2-non-parous	390	89.0	3.0	240
			1-postpartum	368	50.4	0	168
			1-postpartum	182	24.0	0	140
diestrum	7/4[a]	30.0	2-non-parous	846	132.8	4.0	245
			3-parous	665	62.8	2.3	245
	8/1[a]	30.0	5-parous	592	87.4	3.8	300
	8/24[b]	2.0	7-parous	340	143.4	6.8	390
	8/23[a]	40.0	3-parous	356	192.8	12.6	390
proestrum			4-non-parous	386	221.1	17.4	436
	9/9[a]	2.4	8	374	130.8	8.3	540
	9/9[b]	4.0	8	262	118.9	7.6	540
	*9/26[a]	2.4	2	416	118.0	2.6	436
	*9/26[b]	4.0	6	271	78.8	2.6	600
estrus	*10/6[a]	2.0	2	232	47.4	0	172
			1	106	74.6	3.0	256
	*11/26[b]	1.2	5	254	72.9	0	204

[a] Treatment for three days.
[b] Treatment for five days.
* Ovulations occurred in some individuals.

For each collection considered separately, the total number of follicles, the diameter of the largest follicle, the number of follicles with antra, and the volume of the follicle-oocyte system per bat are given in Table 1. Among the mature females the season of the year and the duration of the treatment condition greater variation in the total number of follicles than does the dosage of PU. The recently parous bats of June 18 contain the fewest follicles and the non-parous bats of July 4 contain the greatest number. Within the range used any dosage administered over three days results in more follicles than any dosage administered over five days. The change in the average number of follicles for all of the bats treated with PU as compared with the controls is given in Table 2.

The variations in volume conditioned by season and dosage are summarized in Table 1. The increase in volume is not proportional to dosage, but more vesicular follicles are produced by higher dosages. The percentage change in the volumes of the follicle-oocyte system in the various

TABLE 2. CHANGE IN NUMBER OF FOLLICLES OF BATS TREATED WITH PU COMPARED WITH THE CONTROLS

Collection		3 day treatment dosage R.U.	Percentage change	5 day treatment dosage R.U.	Percentage change
Date	Condition				
6/18	postpartum	28.0	−14.2		
7/4	non-parous	30.0	+31.0		
	parous	30.0	+34.0		
8/1	parous	30.0	+20.2		
8/24	parous	40.0	+ 5.1	2.0	0
9/9	parous	2.4	+ 8.0	4.0	−4.5
9/26	parous	2.4	+53.6	4.0	0
11/26	parous			1.2	+6.6

collections after PU treatment as compared with the controls is given in Table 3.

A comparison of Table 2 with Table 3 indicates that volume is a more significant criterion for ovarian response than number of follicles. On the basis of number of follicles postpartum bats have a negative response to PU whereas volume calculations show an increase in the amount of protoplasm. On August 24 there was no change in the number of follicles after five days treatment, but the size increases were such that the amount of protoplasm was increased 100%.

Within the follicle-oocyte system the growth rate of the oocyte is compared with that of the granulosa in order to find out if PU has a specific effect on the growth rate of one component or the other. For each collection the curves for the treated bats are like those for the controls of that collection (Guthrie, Jeffers and Smith, 1951). The growth rate of the oocyte with regard to the granulosa is affected by the period of the estrous cycle and by inanition but not by PU in the dosages used.

Ovulation: The importance of the stage of differentiation of the follicle-oocyte system is indicated by the ovulations obtained after injections of PU. The ovulations are recorded in Table 4. Neither 2.4 R.U. nor 4.0 R.U. of PU induce any ovulations on September 9, but the same dosages on September 26 produce ovulations in 50% of the animals. The non-

TABLE 3. CHANGE IN THE VOLUME OF FOLLICLES OF BATS TREATED WITH PU COMPARED WITH THE CONTROLS

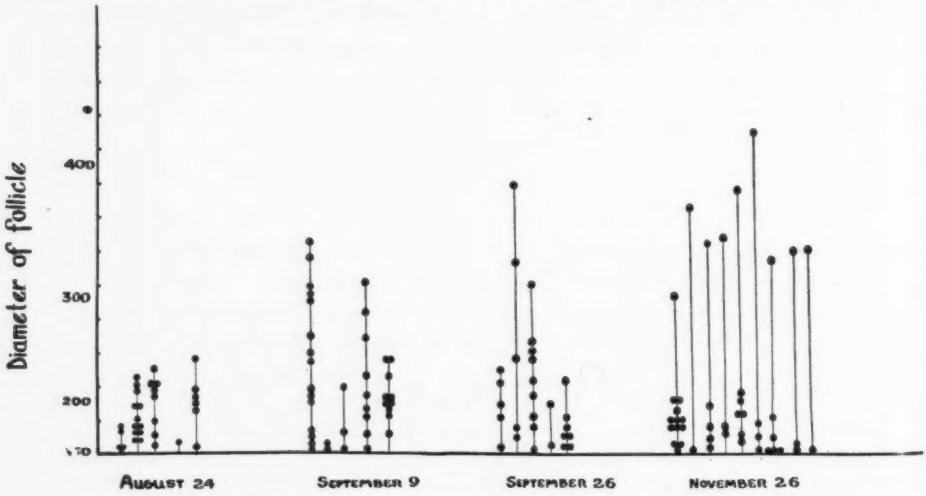
Collection		3 day treatment R.U.	Percentage change	5 day treatment R.U.	Percentage change
Date	Condition				
6/18	postpartum	28.0	+11.2		
7/4	non-parous	30.0	+15.1		
	parous	30.0	0		
8/1	parous	30.0	+14.8		
8/24	parous	40.0	+100.9	2.0	+98.3
9/9	parous	2.4	+75.5	4.0	+72.1
9/26	parous	2.4	+99.8	4.0	+36.4
11/26	parous			1.2	+51.5

TABLE 4. DOSAGES AND OVULATIONS

Collection date	Dosage	Number treated	Number ovulating
September 9	2.4 R.U. in 3 days	8	0
	4.0 R.U. in 5 days	8	0
September 26	2.4 R.U. in 3 days	2	1
	4.0 R.U. in 5 days	6	3
October 6	2.0 R.U. in 3 days	3	1
November 26	1.2 R.U. in 5 days	5	5

ovulating bats of September 26 have preovulatory follicles of normal appearance. No attempt has been made to determine the minimum effective dosage, which varies with the season. During July and August 40.0 R.U. are not adequate to induce ovulations whereas 1.2 R.U. are effective in November. Neither the presence of males nor insemination are factors determining the occurrence of ovulation or volume of the follicle-oocyte system.

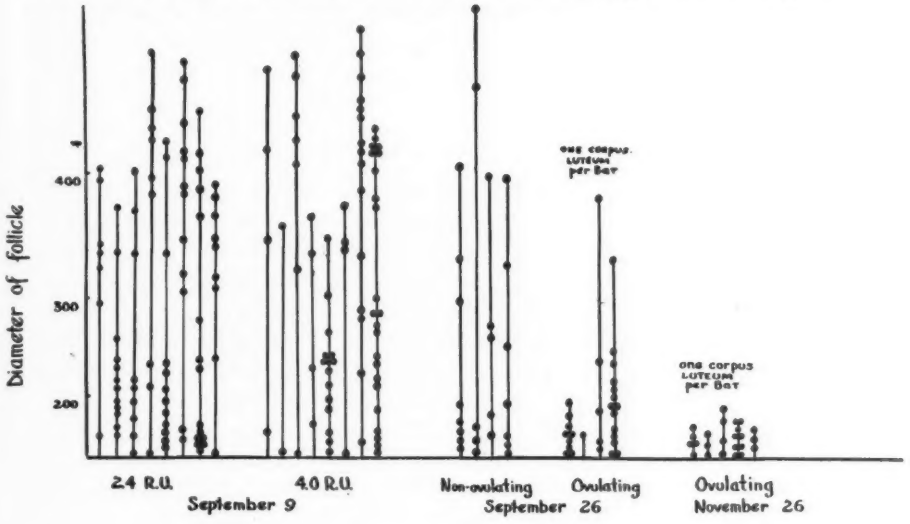
The gradual decrease in the total volume of the follicle-oocyte system is one of the factors that conditions the ease with which ovulation can be induced as the autumn advances. More striking is the appearance at the



DISTRIBUTION of the largest follicles within individual control bats

FIG. 1

beginning of estrus in late September of a lack of continuity in the sizes of follicles in the upper part of the follicle size range. This gap is correlated with the continuous increase from June through November in the diameter of the largest follicle and with the decrease in volume of the follicle-oocyte system which begins in September. The correlation between discontinuity in size and ovulation after PU injection is shown in figures 1 and 2 in which the distribution of follicles having a diameter greater



Distribution of the largest follicles within individual bats treated with PU

FIG. 2

than 150 μ is plotted for individual animals. Ovulation is induced in some of the bats of September 26 but in none of those of September 9, although the largest follicles of the bats of September 9 are as large as those of September 26.

DISCUSSION

Although many investigators find that small follicles are not responsive to gonadotrophins, reactions in non-vesicular follicles have been reported in the rabbit, rat, guinea pig and bat (Desaive, 1935; Lane, 1935; Freed and Coppock, 1936; Guthrie and Jeffers, 1938). In the bat studied, PU stimulates growth in follicles of all sizes. Whether follicles of a given diameter increase in number and volume after PU injection seems to depend not so much upon actual size of the follicle as upon the number and

distribution of the larger follicles present in the bat. Increases in small follicles are most marked in collections in which ovulation or regression of the vesicular follicles occurs.

The induction of ovulation is more difficult in intact bats than in intact rats, mice, and cats in which high dosages of PU, PMS, or hypophyseal extracts result in ovulation at any stage of the estrous cycle (Cole, 1937; Windle, 1939; Burdick and Whitney, 1941; Rowlands, 1944). In the bat superovulation has not been found; ovulation has been induced only during proestrum and estrus when the ovaries have a low total protoplasmic volume, a large follicle and a definite discontinuity in size between the largest follicle and the remainder of the follicles.

CONCLUSIONS

Changes in the follicular apparatus of the ovary of the bat have been observed at different seasons of the year and correlated with the responsiveness to pregnancy urine gonadotrophin by counting the number of growing follicles and calculating the volume of the follicle-oocyte system. Volume is a more significant criterion in determining the character of ovarian response than is the number of follicles. Among mature females the stage of the estrous cycle and the duration of the treatment condition greater variation in follicular response than does the dosage of PU. The induction of ovulation in the bat becomes progressively easier during the prolonged period of submaximal estrus during the autumn and winter. This is correlated with a decrease in the volume of the follicle-oocyte system and with the appearance of a discontinuity in the size of the largest follicle.

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THE INFLUENCE OF PANCREATECTOMY ON THE METABOLIC STATE OF THE ALLOXAN-DIABETIC DOG

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THE most convincing evidence that a hyperglycemic-glycogenolytic factor is produced by the pancreas is the demonstration that removal of the pancreas from the dog previously rendered diabetic by alloxanization (alloxan-diabetic dog) results in a reduction in both the quantity of sugar excreted and the quantity of insulin required to maintain the preoperative level of glycosuria (Thorogood and Zimmerman, 1945; Rodriguez-Candela *et al.*, 1947). Examination of the experimental design that was employed, however, raises doubts as to whether the aforementioned observations actually demonstrated that an amelioration of the diabetic state ensued after pancreatectomy.

The hyperglycemia and glycosuria of the diabetic animal are dependent upon endogenous and exogenous sources of carbohydrate. If the exogenous sources remain constant, then it may be assumed that changes in the degree of glycosuria are due to endogenous factors. Such an assumption was made by Thorogood and Zimmerman (1945) whose experiments were designed to measure the amount of insulin required to maintain a constant glycosuria in animals on a diet which "was originally adjusted so as to maintain a constant body weight." The fallacy of the above assumption, however, lies in the fact that pancreatectomy results not only in a loss of humoral agents but also in a loss of enzymes which are essential for the digestion and absorption of foodstuffs. Consequently, in spite of a constant diet, a reduction in the degree of glycosuria of the alloxanized dog after pancreatectomy may be due to a decrease in the absorption of carbohydrates and carbohydrate precursors rather than to an amelioration of the severity of the metabolic state.

In order to determine the influence of pancreatectomy on the severity

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of the alloxan-diabetic state, it is essential that those effects which are due to the absence of pancreatic humoral agents are distinguished from those which are due to the absence of pancreatic enzymes in the intestinal tract. This may be achieved by evaluating the severity of the diabetic syndrome during periods of fasting before and after pancreatectomy.

METHOD

After a preliminary fast of 48 hours, healthy mongrel dogs were rendered diabetic by the intravenous administration of 100 mgm. alloxan monohydrate per kilogram body weight. As soon as the existence of a significant hyperglycemia and glycosuria was established, the animals were put on a regimen consisting of a constant diet and a quantity of protamine zinc insulin which permitted the maintenance of the body weight in spite of a marked glycosuria. The diet consisted of a mixture of 100 grams Arcady dog ration pellets,* 100 grams horse meat, 4 grams pancreatin, and 5 grams yeast and was supplemented with cod-liver oil. Any food that was not eaten was weighed and the actual daily intake recorded.

At various intervals after the development of a significant hyperglycemia and glycosuria, the animals were fasted for from 4 to 5 days during which period no insulin was administered. At the completion of the fasting period, food and insulin regimen was reinstituted and the body weight allowed to return to the pre-fasting level before any other studies were performed. After adequate controls had been established, the pancreas was removed. Subsequently when the wound was healed and the body weight at the preoperative level, the effect of insulin deprivation and fasting was determined again. During the fasting periods the concentration of sugar (Nelson, 1944) and total acetone bodies (Greenberg and Lester, 1944) of the blood were determined daily as were also the urinary excretion of sugar, total ketone bodies and nitrogen. Only such animals on which studies before and after pancreatectomy were completed are reported herein.

RESULTS

Figure 1 is illustrative of the usual immediate effect of pancreatectomy on the glycosuria of the dog maintained on a constant diet and insulin regimen. With but one exception, there was an immediate reduction in the quantity of sugar excreted after pancreatectomy. It is noteworthy that although the dietary intake and insulin dosage remained unchanged, the body weight also remained constant in spite of the decrease in sugar excretion.

Illustrative of the effect of the pancreatectomy on the endogenous metabolism of different alloxan-diabetic dogs are the data depicted in Figures 2 and 3. Figure 4 illustrates the mean of the data obtained from 7 dogs in which the studies were completed. Although quantitative differences were noted from animal to animal, nevertheless, in every instance, the blood sugar concentration during the fasting period was greater after pancreatectomy. Likewise, after 24 to 48 hours of fasting, the glycosuria was greater after pancreatectomy even though a more intensive glycosuria

* Protein 26%, Fat 5%, Carbohydrate 45.50%.

was commonly observed during non-fasting periods in the same alloxanized dog before pancreatectomy.

After pancreatectomy, the concentration of total ketone bodies in the blood rose more rapidly and to higher levels on fasting than under similar circumstances before pancreatectomy. In order to determine whether the alloxanized dog is capable of forming ketone bodies at a rate similar to

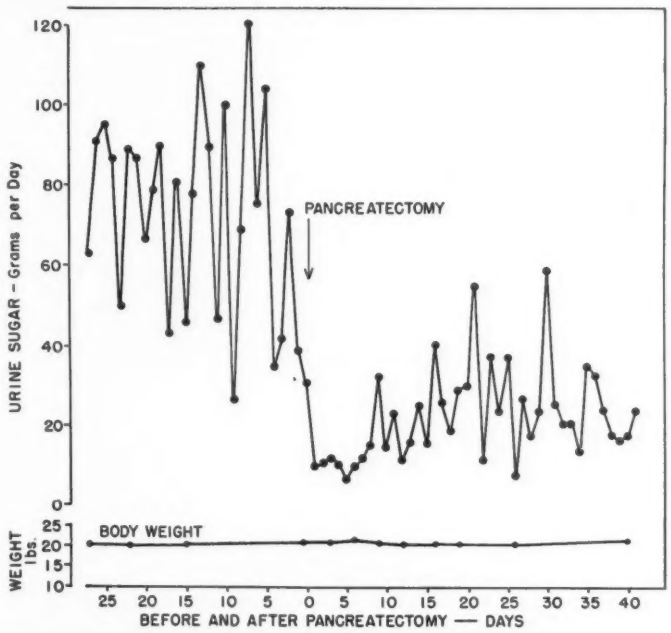

FIG. 1. The effect of pancreatectomy on the glycosuria of a dog rendered diabetic by alloxan. Throughout the period depicted in the figure, the dog ate 385 grams of the diet mixture per day and received 14 units PZI per day.

that observed in the depancreatized dog, phlorhizin was administered to such an animal during a fasting period. The phlorhizin induced a marked glycosuria which was followed by an augmentation of the ketogenesis (Figure 5).

In order to determine the influence of pancreatectomy on the absorption of foodstuffs under our experimental conditions, the carbohydrate and protein content of the feces (Assoc. of Official Agr. Chem., 1945) was determined in the alloxanized and depancreatized dogs. Figure 6 is illustrative of this study and depicts the percentage of the ingested carbohydrate and protein that was excreted with the feces. Whereas there was no significant difference between the carbohydrate loss in the feces of the alloxanized and depancreatized dog, there was a significant difference

in protein loss in that the alloxanized dog excreted 15.1% (S.E. ±2.15) and the depancreatized dog 39.2% (S.E. ±6.25) of the protein intake.

DISCUSSION

The observation that pancreatectomy results in a marked reduction in the quantity of glucose excreted in the urine by the alloxan-diabetic dog on a constant regimen of food and insulin (Figure 1) is in accord with the

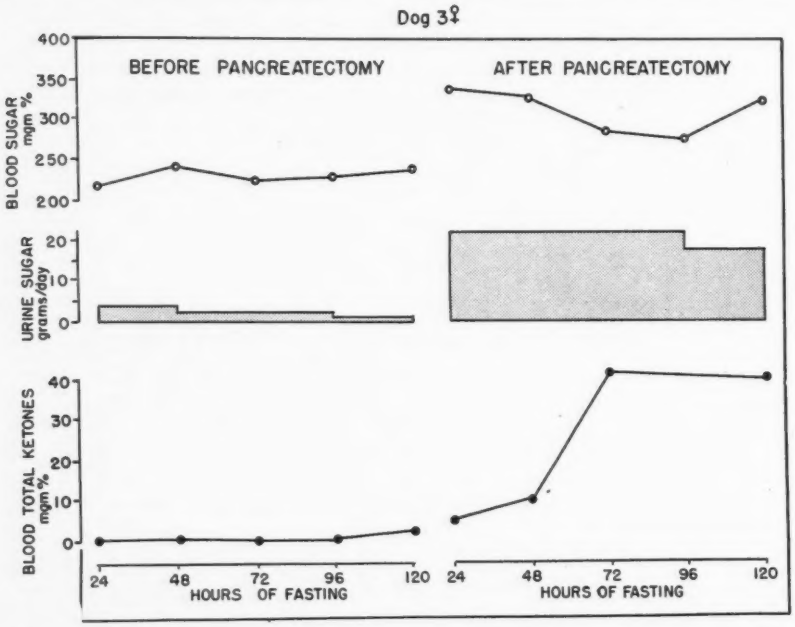

FIG. 2. The effect of a period of fasting and deprivation of exogenous insulin before and after pancreatectomy of an alloxan-diabetic dog. Weight at beginning of fasting periods before and after pancreatectomy was 19 and 17 kilograms respectively. The intake was approximatly 700 grams of the diet mixture per day with a dosage of 10 units of PZI per day.

reports of Thorogood and Zimmerman (1945) and of Rodriguez-Candela and his co-workers (1947). Undoubtedly, as was noted by these investigators, such an animal will require a smaller amount of insulin to maintain that level of glycosuria which was present before pancreatectomy.

With a constant intake of food and an unchanged dosage of exogenous insulin, an increase in body weight should result from a decrease in the quantity of sugar lost in the urine. Actually, the diminished glycosuria of the alloxan-diabetic dog after pancreatectomy results in little or no change

in the body weight of the animal (Figure 1). Therefore, the diminished glycosuria cannot be attributed to an amelioration of the diabetic state, but must be attributed to a diminution in available carbohydrate or carbohydrate precursors from exogenous sources. In accord with this hypothesis is the fact that in spite of the administration of amounts of pancreatin which are adequate for the prevention of the fatty infiltration of the liver and the fatty stools which otherwise occur in the depancreatized dog,

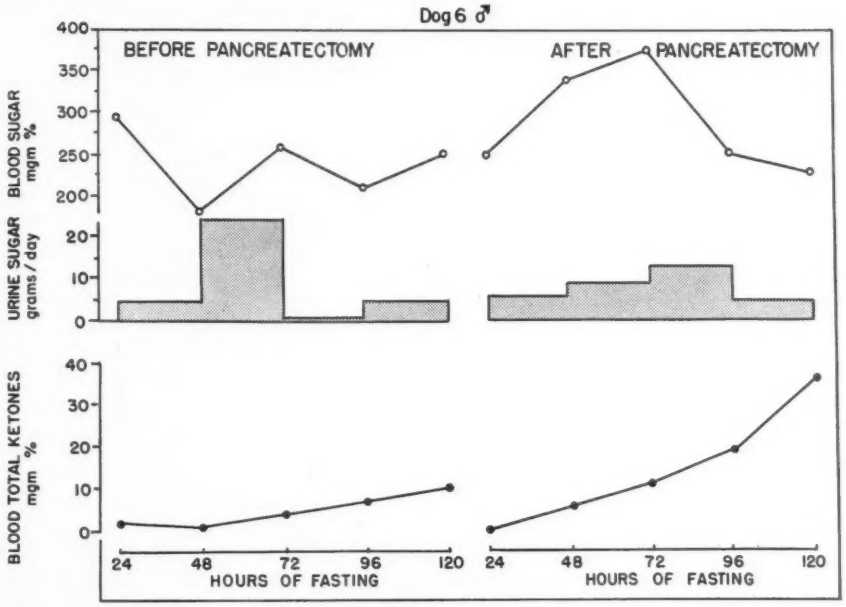

FIG. 3. The effect of a period of fasting and deprivation of exogenous insulin before and after pancreatectomy of the alloxan-diabetic dog. Weight at beginning of fasting periods before and after pancreatectomy was 9.1 and 8.9 kilograms respectively. The intake was 495 grams of the diet mixture per day with a dosage of 10 units PZI per day.

such dogs absorb less protein than do alloxanized dogs (Figure 6). Thus, even if all other factors remained constant, loss of the exocrine function of the pancreas will result in a decreased absorption of protein with a consequent decrease in the quantity of carbohydrate precursors available for gluconeogenesis so that a decrease in glycosuria will ensue. Thus, a dog on an intake of 200 grams of protein per day will absorb approximately 170 grams per day before pancreatectomy and 122 grams per day after pancreatectomy, a difference which is equivalent to approximately 45 grams of carbohydrate. Somewhat similar are the observations in the

hypophysectomized-depancreatized dog where the rate of protein mobilization is decreased. Such animals have a decreased rate of protein mobilization and develop hypoglycemia with starvation but a marked hyperglycemia and glycosuria ensue when large amounts of protein are administered (Soskin, Mirsky, Zimmerman and Crohn, 1935). In the hypophysectomized-depancreatized dog, the decreased gluconeogenesis is due in part to a decreased availability of endogenous protein while in the

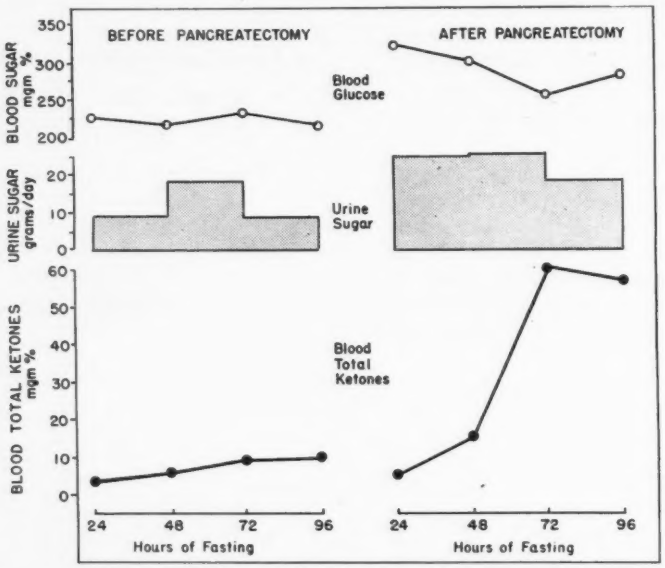

FIG. 4. The effect of a period of fasting and deprivation of exogenous insulin before and after pancreatectomy of alloxan-diabetic dogs. The mean of all data from 7 dogs is depicted.

fed depancreatized alloxan-diabetic dog, the relative diminution of glycosuria is due to a decrease in available exogenous protein.

The hyperglycemia, glycosuria and ketonemia observed during periods of fasting and deprivation of exogenous insulin are dependent upon the state of endogenous metabolism. Consequently, the higher concentration of sugar in the blood, the greater quantities of sugar excreted in the urine, and the more rapid accumulation of ketone bodies in the blood during periods of fasting of the alloxan-diabetic dog after pancreatectomy (Figures 2, 3 and 4) must reflect an aggravation of the severity of the metabolic derangement. At the present time, the only explanation of the mechanism responsible for the intensification of the diabetes that presents itself is that in most instances some of the insulin producing cells of the

pancreas are not destroyed by alloxan and that with pancreatectomy such sources of small amounts of endogenous insulin are eliminated. In accord with this hypothesis is the observation that one alloxan-diabetic dog did not show any reduction in glycosuria after pancreatectomy, presumably because all insulin producing cells had been destroyed by alloxanization.

It is apparent from the preceding that in the fed animal, the decreased absorption of food by the depancreatized dog masks the concomitant

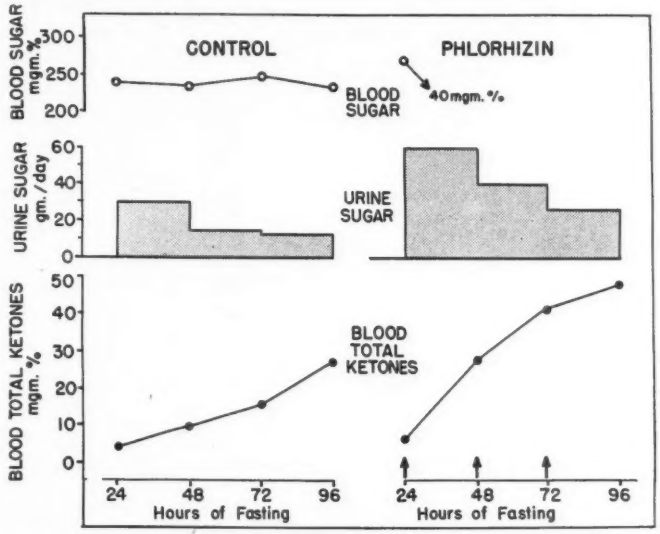

FIG. 5. The influence of phlorhizin on the ketogenesis of the alloxan-diabetic dog. The effect of fasting and deprivation of exogenous insulin was tested and repeated several weeks later when one gram of phlorhizin in oil was injected subcutaneously every day (↑).

intensification of the diabetic state. Therefore, in order to evaluate the true metabolic status of a fed animal, precise measurements of the quantities of food that are actually absorbed must be obtained.

The observation that a minimal rate of ketogenesis occurs in the presence of marked glycosuria in the alloxan-diabetic dog has led to the proposal that such an animal has a decreased susceptibility to ketogenesis because of some property of the hyperglycemic-glycogenolytic factor presumed to be produced by the alpha cells of the Islets of Langerhans (Pincus, 1950). The fact that the alloxan-diabetic animal has the capacity to store large quantities of glycogen (Miller, 1949) and that this capacity would tend to prevent ketogenesis (Mirsky, 1942) has been given little consideration. The observations reported herein suggest that the alloxan-diabetic dog has no diminished susceptibility to ketosis if the glycogen stores of

the liver are depleted consequent to the administration of phlorhizin. It would appear that when the capacity to store glycogen in the liver is disturbed, ketogenesis ensues irrespective of whether or not the pancreas is present.

The demonstration that totally depancreatized men require relatively small quantities of insulin (Gaston, 1948) and that dogs rendered diabetic by the injection of anterior pituitary extracts (Young, 1939), by subtotal pancreatectomy (Dragstedt, Allen and Smith, 1943) or by the administration of alloxan (Thorogood and Zimmerman, 1945) have a diminished

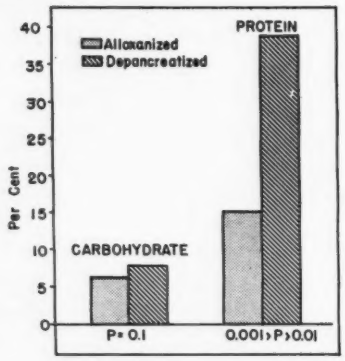

FIG. 6. The effect of pancreatectomy on the percentage of dietary carbohydrate and protein excreted with the feces. The data represent the means computed from daily determinations for 7 days.

requirement for insulin after total pancreatectomy led to the concept that the patient with diabetes mellitus may be suffering in part from the action of a 'hyperglycemic' factor produced by the pancreas. The data reported herein reveal that estimations of insulin requirements which are based on the quantity of sugar excreted by the organism subsisting on some specific diet cannot be utilized as an index of the severity of the endogenous metabolic derangement. Consequently, until more definitive data become available, it is erroneous to interpret the aforementioned observations as indicative of the action of a 'hyperglycemic-glycogenolytic' factor or to postulate that such a factor plays a role in the genesis and maintenance of the metabolic derangement characteristic of diabetes mellitus.

SUMMARY

1. Pancreatectomy produces a diminution of the glycosuria of the dog previously rendered diabetic by alloxan and maintained on a constant dietary intake and insulin dosage.

2. Pancreatectomy produces a decrease in the absorption of proteins

from the intestinal tract as revealed by the increase in the percentage of dietary protein lost with the feces.

3. Pancreatectomy produces an aggravation of the severity of the diabetic state since the hyperglycemia, glycosuria and ketonemia observed during a period of fasting and exogenous insulin deprivation is greater after than before pancreatectomy of the alloxan-diabetic dog.

4. The alloxan-diabetic dog develops a severe ketonemia after phlorhizination and the consequent depletion of hepatic glycogen.

5. The diminished glycosuria following pancreatectomy of the fed alloxan-diabetic dog is attributed to the decreased absorption of carbohydrate precursors which in turn masks the aggravation of the endogenous metabolic derangement.

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GASTRIC SECRETION IN THE ADRENALECTOMIZED RAT

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TUERKISCHER and Wertheimer (1945) using a modification of the technique for the study of gastric secretion first reported by Roe and Dyer (1939) found a significant decrease in gastric secretion in adrenalectomized rats. They particularly emphasized the point that this decrease in gastric secretion could not be accounted for by the surgical trauma imposed upon the animal. A normal volume of secretion was obtained after bilateral adrenal-enucleation. The decrease in gastric secretion following adrenalectomy could be corrected with adrenal cortical extract but not with desoxycorticosterone acetate or normal saline. It seemed important to attempt to confirm these findings. The results herein reported extend and confirm those published elsewhere (Madden and Ramsburg, 1951).

METHOD

Male Sprague-Dawley rats maintained on Purina Dog Chow and water ad lib were preoperatively starved for 24 hours, water being continued. The animals were weighed at the end of this 24-hour starvation period. Their weights appear in Table 1. Using intraperitoneal Nembutal anesthesia, the abdomen was opened, the junction of the pyloric antrum and first portion of duodenum was picked up with a glass hook and ligated. The adrenals were removed from the abdominal approach immediately after the pylorus ligation. Care was taken to minimize hemorrhage. These technics have been fully described in previous publications (Madden *et al.*, 1951). The gastric juice was permitted to accumulate for a 6-hour period subsequent to the time that the animals recovered from the anesthesia sufficiently to respond to stimuli. After this 6-hour period the rats were sacrificed using chloroform. The stomachs were removed and the volume and pH of the gastric juice measured. Cortisone acetate (CA) and desoxycorticosterone acetate[1] (DCA) were injected subcutaneously as was the Aqueous Vehicle No. 1[2] which served as the control.

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[1] Through the generosity of Merck & Company, Rahway, N. J., our desoxycorticosterone acetate was prepared as a microcrystalline suspension (25 mg./cc) in the same menstrum as their cortisone acetate (Cortone) which is supplied commercially.

[2] Merck & Company, Rahway, N. J., generously gave us this menstrum which was used by them to suspend the CA and DCA used in our experiments.

TABLE 1. EFFECT OF CA AND DCA UPON GASTRIC SECRETION IN THE PYLORUS-LIGATED ADRENALECTOMIZED SPRAGUE-DAWLEY RAT

Operation	Therapy	No. rats	Av. wt. (gm.)	Gastric juice	
				Av. vol. (cc.)	Av. pH
	Part 1[1]				
Pylorus-Ligation	Aq. V. No. 1	18	102	8.7 ± .17[2]	1.3
Pylorus-Ligation and Adrenalectomy	Aq. V. No. 1	20	102	5.7 ± .19[2]	1.1
Pylorus-Ligation and Adrenalectomy	CA	23	101	6.4 ± .23[2]	1.3
Pylorus-Ligation and Adrenalectomy	DCA	19	105	6.5 ± .28[2]	1.2
	Second Series[1]				
Pylorus-Ligation	Aq. V. No. 1	10	109	9.2 ± .23[2]	1.3
Pylorus-Ligation and Adrenalectomy	Aq. V. No. 1	20	108	7.4 ± .13[2]	1.1
Pylorus-Ligation and Adrenalectomy	CA	27	105	7.2 ± .04[2]	1.3
	Part 2[3]				
Pylorus-Ligation	Aq. V. No. 1	27	110	9.0 ± .21[2]	1.2
Pylorus-Ligation and Adrenalectomy	Aq. V. No. 1	29	110	5.6 ± .15[2]	1.3
Pylorus-Ligation and Adrenalectomy	CA	28	110	6.4 ± .12[2]	1.1
Pylorus-Ligation and Adrenalectomy	DCA	28	112	6.8 ± .15[2]	1.3
	Second Series[3]				
Pylorus-Ligation	Aq. V. No. 1	15	117	9.2 ± .25[2]	1.2
Pylorus-Ligation and Adrenalectomy	Aq. V. No. 1	27	116	6.6 ± .20[2]	1.3
Pylorus-Ligation and Adrenalectomy	DCA	31	116	7.9 ± .16[2]	1.1
	Part 3[4]				
Pylorus-Ligation	Aq. V. No. 1	31	122	9.9 ± .18[2]	1.1
Pylorus-Ligation and Adrenalectomy	Aq. V. No. 1	33	123	6.5 ± .15[2]	1.2
Pylorus-Ligation and Adrenalectomy	CA	33	122	8.2 ± .17[2]	1.3
Pylorus-Ligation and Adrenalectomy	DCA	32	121	6.6 ± .18[2]	1.2
	Second Series[4]				
Pylorus-Ligation	Aq. V. No. 1	14	103	8.5 ± .24[2]	1.1
Pylorus-Ligation and Adrenalectomy	Aq. V. No. 1	29	107	6.1 ± .21[2]	1.2
Pylorus-Ligation and Adrenalectomy	CA	29	105	7.3 ± .19[2]	1.3

[1] Sixteen hours prior to operation each rat received 3.75 mg. of CA or DCA or .15 cc. of Aq. V. No. 1.

[2] Probable error of the mean result.

[3] Sixteen and 1 hour prior to operation each rat received 1.9 mg. of CA or DCA or .075 cc. of Aq. V. No. 1.

[4] One hour prior to operation each rat received 3.75 mg. of CA or DCA or .15 cc. of Aq. V No. 1.

RESULTS

Table 1 is divided into 3 parts and each part into two series. In Part 1, 3.75 mg. of either CA or DCA was administered 16 hours prior to operation and in Part 2, the dosage of CA or DCA was divided into equal amounts (1.9 mg.) and given 16 and 1 hour prior to operation. In Part 3, the 3.75 mg. of CA or DCA was given as one dose 1 hour prior to operation. The second series in each part was done to confirm the most important finding of each part. These results are presented separately since each series was done with its own simultaneous controls. In each experiment, a result was not considered significant unless the difference between two means was at least 3.5 times greater than the probable error of the difference.

Part 1 clearly demonstrates the sharp fall in gastric juice volume after adrenalectomy. CA and DCA did not increase the gastric juice volume significantly when given to adrenalectomized pylorus ligated rats 16 hours

prior to operation. The second series of Part 1 confirmed the inability of CA to increase the volume of gastric secretion when given under these conditions.

Part 2 demonstrates again the fall of gastric secretion after adrenalectomy. DCA given in divided doses 16 and 1 hour prior to the pylorus ligation-adrenalectomy procedure resulted in an increase in gastric juice volume when compared to similar animals not receiving DCA. This increase was highly significant statistically, the difference between the means being 5 times the probable error of this difference. Similar rats receiving CA in divided doses also showed an increase in gastric juice volume. However the increase was smaller than with DCA and was not quite significant statistically, the difference between the means being only 3 times its probable error. The second series of Part 2 confirmed the positive effect of DCA on gastric secretion under these circumstances.

When the entire dose of steroids was give 1 hour prior to operation, Part 3, CA caused a highly significant increase in gastric juice volume while DCA was ineffective. The second series of Part 3 confirmed the finding that CA was active in partially restoring the gastric juice volume to the control level.

It should be noted that neither CA nor DCA was able to prevent more than about 50 per cent of the decrease in gastric juice volume which occurred in adrenalectomized pylorus-ligated rats when given in the most favorable dosage time schedule found in our experiments.

No significant changes in the pH of the gastrie contents were noted in any of these experiments.

DISCUSSION

These results demonstrate clearly the marked influence of the adrenal glands on gastric secretion under the conditions of our experiments. We have published results elsewhere (Madden and Ramsburg, 1951) which show that either adrenal cortical extract or saline will partially prevent depression in gastric secretion following adrenalectomy. The fact that these substances are active plus the fact that either DCA or CA, under certain conditions, will also partially prevent this effect of adrenalectomy, suggest that the adrenal secretions constitute an important controlling influence on the biological phenomena which are fundamental in gastric secretion. These findings are in harmony with the recent work of Spiro *et al.* (1950) in which they showed that adrenocorticotrophic hormone markedly increased the excretion of uropepsin in normal individuals. This hormone did not increase the low uropepsin excretion found in patients with adrenal insufficiency although Compound E (Cortisone) did.

Our results confirm those of Tuerkischer and Wertheimer (1945) with

respect to decreased gastric secretion after adrenalectomy and prevention of this effect with adrenal cortical extract. Our results differ in that they found saline and DCA to be inactive in their tests. Saline was very active in our experiments (Madden and Ramsburg 1951) while DCA was active when given according to a certain dosage-time schedule as shown in the present experiments.

The mechanism by which the adrenal influences gastric secretion is not known. It is possible that the adrenal steroids may have a direct effect on the secretory process in the gastric mucosa. However, this seems quite unlikely in view of the work of Davenport and Chavre (1950). They have shown that stomachs removed from normal and adrenalectomized mice secrete acid at the same rate when incubated in vitro. Furthermore neither desoxycorticosterone nor cortisone increased gastric secretion when added to the incubation fluid. The adrenal steroids are known to have marked effects on salt and water metabolism as well as many other physiological processes important to gastric secretion. We have previously shown (Madden *et al.*, 1951) that gastric secretion in the pylorus-ligated rat is quite sensitive to changes in body hydration. The adrenal may, therefore, exercise its influence on gastric secretion, in part at least, by altering the amount of water and electrolytes available for the secretory process. This may explain why CA and DCA produced effects on gastric secretion of variable magnitude depending on the time-dosage relationship to the experimental period. Different rates of absorption and excretion of the steroids, differences in the relative effectiveness of the two compounds in changing salt and water metabolism and different durations of action may also be involved.

SUMMARY

Adrenalectomy significantly reduced the volume of gastric secretion in pylorus-ligated rats.

Desoxycorticosterone acetate and cortisone acetate were effective in partially preventing this effect of adrenalectomy when given under certain conditions.

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EFFECT OF HYPOPHYSIAL STALK SECTION ON ADENOHYPOPHYSIAL FUNCTION*

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ALTHOUGH neural regulation of neurohypophysial secretion is generally accepted, the mechanism of adenohypophysial secretion is undetermined. Direct neural control of thyrotrophic (Uotila, 1939; Brolin, 1946) and gonadotrophic secretion (Brooks, 1940) has been postulated, but more recent studies postulate that neurohumors regulate secretion of some adenohypophysial hormones (Markee, Sawyer and Hollinshead, 1946; Harris, 1948; Hume and Wittenstein, 1950).

For adrenocorticotrophic hormone (ACTH) secretion, there is special *a priori* reason to suspect neural regulation. Secretion of this hormone is accelerated by a wide variety of "stress conditions" (heat, cold, anoxia, pain, toxins, etc.). These stimuli, apparently unrelated, share the common property of exciting sympathetic nervous discharge and liberating epinephrine. For this reason, epinephrine has been proposed as the excitatory link between stress exposure and ACTH secretion (Vogt, 1944; Long, 1947). Direct neural control of the adenohypophysis via the hypophysial stalk is an equally plausible possibility.

In this paper is reported the effect of stalk section on ACTH secretion in response to a standard "stress" stimulus, viz. cold and fasting. Adrenal cholesterol depletion is used as an index of ACTH secretion. Also observations on growth, water metabolism, and hypophysial and gonadal structure of "stalk-sectioned" animals are reported.

MATERIALS AND METHODS

Male guinea pigs (400–600 gm.) were fed a stock diet supplemented daily with carrots and ascorbic acid (50 mgm.). All animals were kept at a constant environmental temperature of 29° C. for at least two weeks prior to use. Body weight, urine volume and urine specific gravity were recorded daily.

The animals were divided into four experimental groups: an operated group and an unoperated group, sacrificed after an 18-hour fast at 29° C. and two comparable groups, sacrificed after an 18-hour fast at 5° C. Water was supplied to all animals during the test period. The adrenal glands and gonads were removed immediately from sacrificed ani-

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mals, and preserved for cholesterol determinations and histological examination, respectively. The carcasses were perfused with saline followed by 10 per cent formalin. The brain was further fixed in formalin, sectioned serially, and stained with thionin or Masson's stain. Adrenal cholesterol content was determined by a slight modification of the Schoenheimer-Sperry technique.*

In the operated animals, the pituitary stalk was exposed aseptically by the transtemporal route and severed with a hooked knife. A spatula was then inserted between the brainstem and hypophysis to insure complete transection.

RESULTS

Criteria of completeness of stalk section. Previous studies of the effect of stalk section on hypophysial function have often yielded conflicting results. In some instances, discrepant results are probably attributable to incomplete division of the stalk. In the present experiments, complete transection of the pituitary stalk was verified by both functional and histological studies. Diabetes insipidus is a well known consequence of stalk section, but may be absent if the level of transection is close to the gland (Fisher, Ingram and Ranson, 1938). Therefore, absence of polyuria is not proof of incomplete section but its occurrence is good evidence of complete transection. Moreover, because diabetes insipidus does not develop in the absence of a functional adenohypophysis (Fisher, Ingram and Ranson, 1938), its occurrence is assurance that the adenohypophysis has not been destroyed by ischemia or accidental operative damage.

All of the operated animals used in the present experiments had a well marked diabetes insipidus; maximal pre- and postoperative urine outputs are recorded in Table 1. Figure 1 shows a typical record of diabetes in an operated animal. The bimodal course of the diuresis has been described for other species (Fisher, Ingram and Ranson, 1938).

In addition, transection of the stalk was confirmed in some animals by microscopic examination of serial sections through the lesion. Figure 2 shows a sagittal section through the brainstem and hypophysis of a guinea pig which was sacrificed 71 days after stalk section. A comparable section from an unoperated guinea pig is also presented. In the operated animal, the cleavage of the stalk is apparent. The clear, poorly-staining pars nervosa has almost completely disappeared and is represented only by elongated glial-like cells whose linear arrangement follows the course of the degenerated nerve fibers. The posterior lobe as a whole, however, is not markedly shrunken; the space vacated by the atrophied pars nervosa is filled with loosely arranged vacuolated tissue composed of irregularly shaped cells with oval nuclei and multiple nucleoli. The increased cellular-

* We are indebted to Dr. Jay Tepperman, Department of Pharmacology, State University of New York at Syracuse, for helpful suggestions and advice on methods of adrenal cholesterol determination.

TABLE 1. EFFECT OF STALK SECTION ON THE DISCHARGE OF ACTH IN RESPONSE TO COLD EXPOSURE

No.	Body wt. (gms.)	Gland wt. (mgms.)	Adrenal cholesterol (Gm./100gm. fresh gland)
Group 1. Intact animals fasted 18 hours at 29° C.			
33	760	267.6	3.77
34	780	211.8	4.40
35	990	355.9	3.97
36	910	361.7	3.71
37	700	210.0	4.76
38	850	281.9	4.34
54	650	148.3	4.19
55	700	143.2	4.72
56	700	194.7	5.28
Mean	782	241.7	4.35
S. E.			0.18
Group 2. Intact animals fasted 18 hours at 5° C.			
60	630	133.0	2.84
61	635	119.4	3.62
62	805	239.7	4.11
63	640	120.3	3.59
64	755	206.1	1.57
65	975	227.6	3.38
141	840	258.6	1.93
142	640	273.0	3.21
143	770	313.3	1.94
144	695	157.8	2.05
145	760	317.2	0.98
Mean	741	215.1	2.66
S. E.			0.30

No.	Body wt. (gms.)	Gland wt. (mgms.)	Adrenal cholesterol (gm./100 gm. gland)	Max. urine vol. (cc./day)	
				Before operation	After operation
Group 3. Stalk-sectioned animals fasted 18 hours at 29° C.					
120	790	178.1	5.01	30	292
121	900	159.6	5.25	42	78
122	900	177.7	3.72	35	275
123	750	221.2	2.87	35	157
126	820	185.4	3.21	38	48
Mean	832	184.4	4.02	36	170
S. E.			0.48		
Group 4. Stalk-sectioned animals fasted 18 hours at 5° C.					
105	785	192.9	1.68	38	153
106	873	453.8	1.67	46	261
107	980	354.4	1.45	42	256
108	825	397.4	1.48	48	340
112	1005	296.5	2.64	47	150
113	810	245.0	3.31	56	116
115	780	391.0	2.52	42	131
Mean	865	333.0	2.17	45	201
S. E.			0.26		

ity of the posterior lobe after stalk section has been previously noted (Cushing and Goetsch, 1910; Keller, Noble and Hamilton, 1936; Mahoney and Sheehan, 1936; Brooks, 1938; Fisher, Ingram and Ranson, 1938; Uotila, 1939), but the origin of the cells is undecided. In Masson-stained sections, they resemble strikingly the cells of the pars intermedia in unoperated animals.

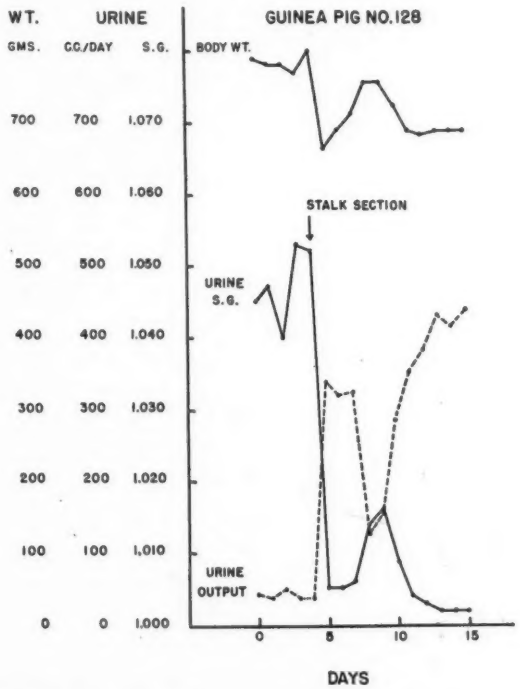

FIG. 1. Daily pre- and postoperative records of body weight and urine specific gravity and volume in guinea pig 128. Note that in the permanent phase, the daily urine output was approximately 70 per cent of the body weight.

Finally, retrograde degeneration of the supraoptic nucleus was sought as a sign of complete stalk transection. Figure 3 shows the atrophied nucleus in an operated animal and a similar section from an unoperated animal for comparison.

Effect of stalk section on secretion of ACTH. Two types of experiment were conducted to determine the effect of stalk section on ACTH secretion. The first type was designed to determine whether severing the stalk significantly alters adrenal cholesterol in animals kept under non-stressful conditions, i.e. at 29° C. The second type tested the capacity of the

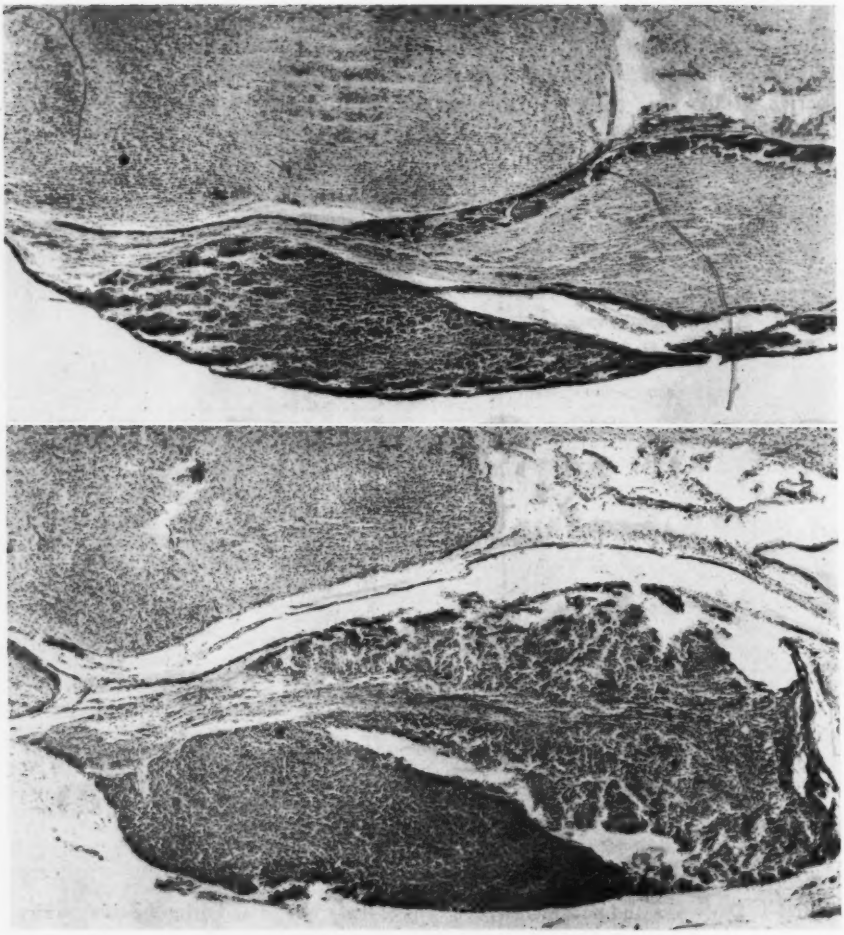

FIG. 2. Upper. Sagittal section of hypophysis and brainstem of an unoperated guinea pig (No. 143). Note stalk at lower left and fibers coursing over the pars distalis into the expanded, poorly-staining pars nervosa. The dark-staining tissue enveloping the pars nervosa is pars intermedia. Thionin.

Lower. Sagittal section of hypophysis and brainstem of guinea pig 112, sacrificed 71 days after stalk section. Note interruption of stalk and almost complete degeneration of pars nervosa. The dark-staining cells filling the posterior lobe resemble pars intermedia cells. Thionin.

denervated hypophysis to secrete ACTH in response to stress (18 hours exposure to 5° C.) as measured by adrenal cholesterol depletion. Of the stalk-sectioned animals, only those which developed a clear-cut diabetes insipidus were used and all were allowed a sufficient postoperative period (27–75 days) to regain their preoperative weights.

The results of the first type of experiment are shown in Table 1. Group 1 consisted of nine intact animals, maintained at 29° C., which were sacrificed after an 18-hour fast. Group 3 consisted of five stalk-sectioned animals sacrificed under identical conditions. The mean adrenal cholesterol content

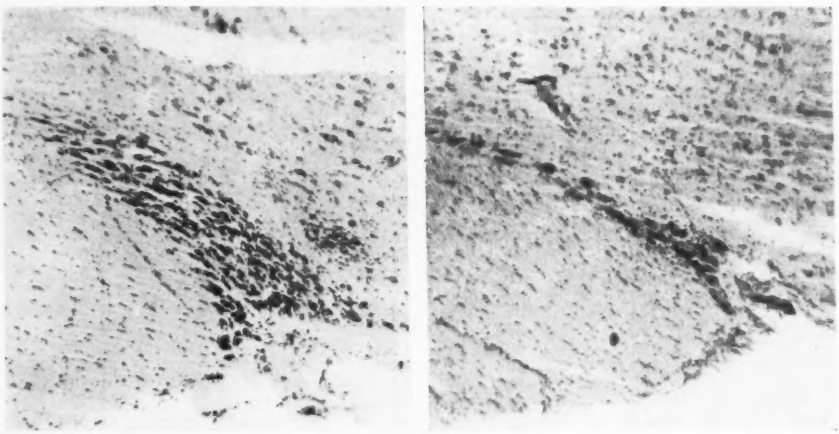

FIG. 3. Left. Sagittal section through the supraoptic nucleus of unoperated guinea pig (No. 143). Thionin.

Right. Comparable section from guinea pig 106 sacrificed 43 days after stalk section. Note decrease of cell density and swelling and pyknosis of remaining cells. Thionin.

of the intact animals (Group 1) was 4.35 gm./100 gm. glandular tissue; for the stalk-sectioned animals (Group 3), 4.02 gm. Although the adrenal cholesterol level was slightly lower in the operated group, the difference proved insignificant when tested by Fisher's *t* test. These experiments demonstrate, therefore, that section of the hypophysial stalk does not significantly alter adrenal cholesterol content.

Table 1 also shows the results of experiments designed to test the response of the intact and denervated hypophysis to cold exposure. Group 2 consisted of eleven intact guinea pigs sacrificed after an 18-hour fast at 5° C. Group 4 contained seven stalk-sectioned animals sacrificed under the same conditions. The mean adrenal cholesterol content of the intact (Group 2) animals was 2.66 gm./100 gm. fresh gland. Comparison with the Group 1 animals indicates that the cold exposure caused a 38.9 per

cent decrease of adrenal cholesterol in the intact animals. The mean adrenal cholesterol content of the operated animals exposed to cold (Group 4) was 2.17 gm./100 gm., which represents a 46.0 per cent reduction under values in Group 3. These data, also shown graphically in Figure 4, demonstrate that cold exposure markedly reduces adrenal cholesterol content in both intact and stalk-sectioned animals. It is therefore concluded that

TABLE 2. EFFECT OF STALK SECTION ON TESTES WEIGHT

Group	No.	Days after operation	Testes wt. (gm.)	Body wt. (gm.)	Testes wt. (gm.) / Body wt. (kg.)
Intact Animals	141	—	4.277	840	5.092
	142	—	3.712	640	5.800
	143	—	3.955	770	5.136
	144	—	2.857	695	4.111
	145	—	4.394	760	5.782
Mean			3.839	741	5.184
S. E.			0.273		0.308
Stalk-Sectioned Animals	105	75	3.998	785	5.093
	106	44	3.588	873	4.110
	107	42	6.270	980	6.398
	108	54	4.963	825	6.016
	112	71	4.402	1005	4.380
	113	64	3.608	810	4.454
	115	57	3.822	780	3.618
	120	39	3.336	790	4.223
	121	48	3.005	900	3.339
	122	39	3.933	900	4.370
	123	27	3.497	750	4.663
	126	27	3.923	820	4.784
	128	82	3.766	805	4.678
	131	74	4.288	915	4.686
Mean			4.029	853	4.629
S. E.			0.215		0.217

factors other than stalk-mediated nerve impulses cause the release of ACTH in response to cold exposure.

Effect of stalk section on the secretion of gonadotrophic hormones. Testes weights of 5 intact and 14 stalk-sectioned animals are given in Table 2. The average total weight of both testes was 3.839 gm. in the intact and 4.029 gm. in the stalk-sectioned animals. Expressed in gm./kg. body weight (last column, Table 2), the average testes weight was 5.184 in intact and 4.629 in stalk-sectioned animals. These differences are not satistically significant.

Histological examination of the testes of the stalk-sectioned animals showed mature spermatozoa as well as successive stages of spermatogenesis in the seminiferous tubules. The epididymides of the stalk-sectioned animals showed no atrophy of the epithelium and contained mature spermatozoa. The seminal vesicles appeared normal in size and structure.

TABLE 3. EFFECT OF STALK SECTION ON GROWTH

No.	Initial body wt. (gm.)	Final body wt. (gm.)	Days	Daily wt. gain (gm.)
Group 1. Unoperated animals				
111	830	865	9	3.9
112	585	865	65	4.3
113	580	755	41	4.3
114	520	745	40	5.6
115	715	740	8	3.1
116	500	530	7	4.3
117	555	590	19	1.8
120	685	750	13	5.0
123	745	790	12	3.8
134	770	810	7	5.7
Mean	—	—	—	4.2
S. E.				0.37

Group 2. Unoperated animals, fasted 12 hours and anesthesized with sodium pentobarbital (40 mg./kg.)

No.	Body wt. before treatment (gm.)	Body wt. 1 day after treatment (gm.)	Body wt. loss (gm.)	Final body wt. (gm.)	Days	Daily wt. gain (gm.)
113	755	730	25	745	4	6.2
116	535	525	10	610	10	8.5
122	890	800	90	890	10	9.0
123	790	785	5	820	6	5.8
Mean	742	710	32.5	—	—	7.4
S. E.						0.8

Group 3. Stalk-sectioned animals, fasted 12 hours prior to the operation which was done under sodium pentobarbital anesthesia (40 mg./kg.)

No.	Body wt. before operation (gm.)	Body wt. after operation (gm.)	B. W. loss (gm.)	Body wt. at 10th day (gm.)	Daily wt. gain in 10 days (gm.)	Final body wt. (gm.)	Days after 10th day	Daily wt. gain after 10th day (gm.)
107	850	800	50	900	10.0	1000	18	5.6
112	865	755	110	840	8.5	925	15	5.7
113	745	655	90	700	4.5	740	9	4.5
120	750	630	120	695	7.0	780	24	3.5
121	820	690	120	740	5.0	785	16	2.8
123	785	665	120	740	7.5	780	16	2.5
126	820	770	50	820	5.0	860	16	2.5
129	685	600	85	680	8.0	745	7	9.4
130	680	645	35	715	7.0	740	6	4.2
132	780	665	115	780	11.5	800	4	5.0
Mean	778	687.5	89.5	761	7.4	—	—	4.6
S. E.					0.71			0.66

In one operated animal (No. 104, not included in the Table), the testes were definitely atrophic (1.538 gm.). In this animal, cold exposure did not significantly reduce the adrenal cholesterol content (3.94 gm./100 gm.). Gross and microscopic examination revealed marked adenohypophysial atrophy. The lesion in the stalk was more extensive than in the other animals and had invaded the median eminence. The results indicate that stalk section unless complicated by secondary adenohypophysial atrophy does not impair gonadotrophin secretion insofar as gonadal weight and structure are reliable indices of secretion.

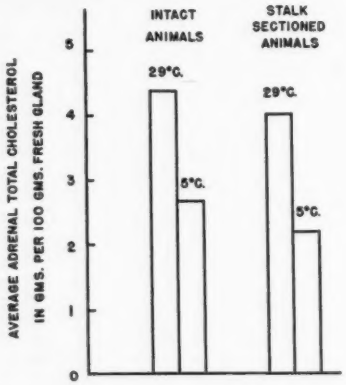

FIG. 4. Effect of cold exposure on adrenal cholesterol content in normal and stalk-sectioned guinea pigs.

Effect of stalk section on growth. Fourteen intact and 10 stalk-sectioned animals were studied in this experiment. Fairly large animals past the age of rapid growth were used because operative procedures proved easier in larger animals. However, none of the animals had attained full growth and some kept for long periods of time continued to grow symmetrically until body weight was doubled. In none of the operated animals was growth permanently impaired. On the first postoperative day the body weight decreased 35–120 gm. (Table 3). This loss apparently resulted partly from the 18-hour preoperative fast but more from the severe diuresis while the animal, recovering from the anesthesia, was unable to drink enough water to prevent dehydration. Unoperated control animals, fasted and anesthetized for comparable periods of time lost only 5–90 gm. (Table 3). The operated animals required 10–30 days to regain their preoperative weights, the time varying with the severity of diuresis.

Because all the stalk-sectioned animals lost considerable weight immediately after operation, the first ten postoperative days were taken arbitrarily as a recovery period. During the recovery period, the average

increase in body weight of stalk-sectioned animals was 7.4 gm. per day, which agrees perfectly with the average increase of 7.4 gm. per day during the recovery period of the control animals (Groups 2 and 3 in Table 3). The average daily weight gain after the recovery period was 4.6 gm. in the stalk-sectioned and 4.2 gm. in the intact animals (Groups 1 and 3 in

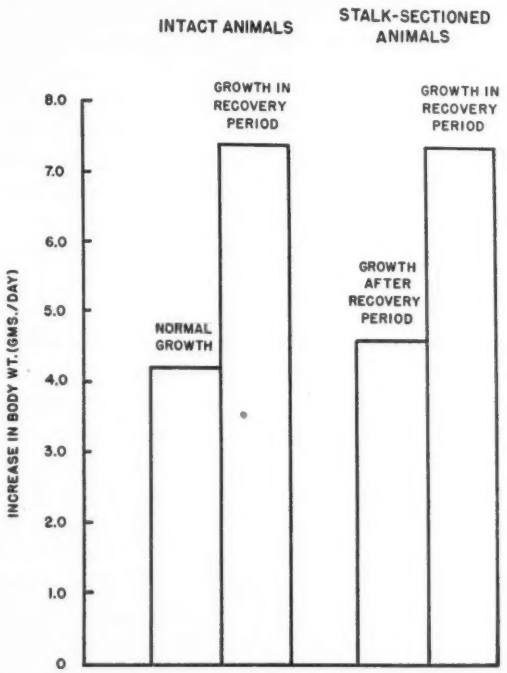

FIG. 5. Mean growth rate of normal and stalk-sectioned guinea pigs. The right hand columns show growth rates during period of recovery from acute weight loss (fasting and anesthesia); the left hand columns, growth rates after recovery of preoperative weights.

Table 3). The similarity of growth rate in these two groups is graphically illustrated in Figure 5.

DISCUSSION

The results indicate that cold-evoked depletion of adrenal cholesterol does not depend upon intact hypothalamo-hypophysial pathways. Our results thus confirm by different methods those of Cheng *et al.* (1949a), which were published while this work was nearing completion. In rats, they found that the decrease in adrenal ascorbic acid resulting from intravenous injection of histamine was not affected by sucking away the posterior lobe and variable amounts of the anterior lobe, thus presumably destroying

the stalk. They cautiously pointed out that the dose of histamine used (1 mgm./100 gm. of body weight) caused a nearly maximal reduction in adrenal ascorbic acid, so that defects in fine regulation of ACTH secretion resulting from the operation might have been masked. Our experiments are subject to the same criticism, but have the advantage that the stress employed is one known to be associated with activation of the hypothalamic centers for temperature regulation. Neural activation of ACTH secretion might thus logically be suspected as a part of the integrated neural response to cold exposure. "Chemical stress" such as intravenous histamine injection might well act directly. However, the similar results obtained in the two experiments indicate that an intact stalk is not essential to the hypophysial response to either stress stimulus. Hume and Wittenstein (1950) also noted that stalk section in dogs does not prevent the eosinopenic response to "trauma, insulin, chemical irritants, and epinephrine."

Our experiments do not rule out conclusively the role of the hypothalamo-hypophysial portal system in ACTH secretion (Harris, 1948). Although this system is undoubtedly interrupted by stalk section, the portal vessels are said to regenerate rapidly (Harris, 1948). Since the animals were not sacrificed until 27 to 75 days after operation such regeneration may well have occurred. However, histological examination revealed little, if any, direct connection between the hypothalamus and the hypophysis in our stalk-sectioned animals. For example in the section shown in Figure 2 there is no direct connection between the hypothalamus and the gland. This section is from guinea pig 112 which was sacrificed 71 days after operation and responded to cold exposure with a marked decrease in adrenal cholesterol (Table 1). Our results thus suggest that the portal system is unessential to activation of the adenohypophysis. In a recent paper, Cheng *et al.* (1949b) reported that adenohypophysial tissue transplanted to the anterior chamber of the eye secretes ACTH in response to histamine injection. Fortier and Selye (1949) obtained similar results using cold and surgical trauma as stresses. Thus these findings combined with our own seem to eliminate the hypothalamo-hypophysial portal system as an essential link between stress exposure and ACTH secretion.

The effect of stalk section on the gonads has been frequently studied but with divergent results. Gonadal atrophy has been both affirmed (Harris, 1937; Brolin, 1946) and denied (Uotila, 1939; Brooks, 1940). In our experiments uncomplicated stalk section did not alter gonadal size or structure. In only one operated animal (No. 104) was there clear cut gonadal atrophy but this was explained by secondary hypophysial atrophy. The lesion to the stalk, which invaded the median eminence, was apparently large enough to cause ischemic atrophy of the hypophysis. Dey

(1943) reported that lesions of the median eminence in female guinea pigs caused gonadal atrophy and cessation of reproductive cycles although stalk section near the gland did not. Our experiments thus suggest that gonadal atrophy results from stalk section only when there is secondary reduction of adenohypophysial tissue.

In guinea pig 104 adrenal cholesterol did not decrease following cold exposure indicating that secretion of ACTH as well as gonadotrophins was impaired. On the other hand, the growth rate was unaltered and the animal had a marked diabetes insipidus which does not occur in a totally hypophysectomized animal. Thus the secretory functions of the partially atrophied adenohypophysis may be dissociated.

The present experiments, in agreement with those of most other investigators (Brooks, 1938; Fisher, Ingram and Ranson, 1938; Uotila, 1939; Rasmussen, 1940), indicate that interruption of the hypophysial stalk does not impair growth. However, Cheng *et al.* (1949a) reported impaired growth in stalk-sectioned rats. In their experiments the stalk was not selectively sectioned but the posterior lobe and part of the anterior lobe were removed by suction. This reduction of adenohypophysial tissue rather than denervation could have caused growth failure. Reduction of functional tissue may also explain failure of growth in hypophysectomized animals with intraocular hypophysial transplants (Cheng *et al.*, 1949b; McDermott *et al.*, 1950).

SUMMARY AND CONCLUSIONS

The effect of hypophysial stalk section on adenohypophysial function was studied in guinea pigs. Complete stalk section was indicated by the occurrence of diabetes insipidus in all operated animals. In addition, complete section was often verified by histological examination of the stalk and the supraoptic nuclei. Secretion of ACTH in response to cold stress was tested by measuring adrenal cholesterol content. Body weight, gonad weight and gonad histology gave information on the secretion of growth hormone and gonadotrophins. The results indicated that secretion of ACTH, growth hormone and gonadotrophins does not depend on an intact hypophysial stalk.

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THE EFFECT OF PURIFIED GROWTH HORMONE ON UREA FORMATION IN NEPHRECTOMIZED RATS[1]

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THE administration of a partially purified preparation of anterior pituitary growth hormone to nephrectomized rats was followed by a considerable decrease in the rate of urea production seen after the intravenous administration of protein hydrolysate (Russell and Cappiello, 1949). Highly purified growth hormone preparations have now been employed in similar circumstances. The previous observations have been confirmed in two series of experiments, and some information has been obtained on the relationship of dosage of growth hormone to the effect obtained.

METHODS

The experimental procedure employed was the same as that described previously (Russell and Cappiello, 1949): Young male rats, fasted 18 hours, were nephrectomized, and 3 to 3½ hours later the rate of increase of urea in the blood was determined after the administration either of casein hydrolysate or of physiological salt solution. In the work reported here, two separate series of experiments were performed some months apart. The 4 to 6 animals which were observed in parallel on any one day included in each instance both control and growth hormone treated rats.

The casein hydrolysate which was injected, prepared as described previously, contained in one set of experiments 12.1 mg. of nitrogen per ml. and in the other, 12.9 mg. of nitrogen. One ml. of amino acid solution, or 0.5 ml. of saline, was given intravenously per 100 gm. body weight. Blood samples of 0.2 ml. each were taken just before the injection of the hydrolysate or saline, and again at 1 and at 3 hours afterwards. The urea content of the blood was measured by a modification of the method of Archibald (1944). The growth hormone preparations were made as described by Wilhelmi, Fishman, and Russell (1948). Both of the samples used appeared to be homogenous by electrophoretic analysis. Freshly prepared solutions of the hormone were administered intraperitoneally 1 to 1½ hours before the observations were begun.

OBSERVATIONS

The results of these experiments are presented in Table 1. It may be seen that the rate of urea formation observed in the hour after the administra-

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TABLE 1. EFFECTS OF PURIFIED GROWTH HORMONE ON THE BLOOD UREA OF NEPHRECTOMIZED RATS

	No. of observ.	Increase in blood urea N mg. per cent per hr.		
		1st hour		2nd and 3rd hours
		Increase	Difference from untreated	
Exp. 1. (G.H. prep. 260)				
1. Saline injection				
A. Untreated	5	3.3±0.48		4.2±0.31
B. Growth hormone 1 mg. per 100 gm.	4	3.0±0.53	−0.3±0.72	3.0±0.38
2. Amino acid injection (12.1 mg. N per 100 gm.)				
A. Untreated	5	11.1±0.34		3.7±0.33
B. Growth hormone 1 mg. per 100 gm.	6	7.2±0.54	−3.9±0.58	3.9±0.36
3. Increase with amino acid injection over saline control series				
A. Untreated	10	+ 7.8±0.59		−0.5±0.46
B. Growth hormone	10	+ 4.2±0.69		+0.9±0.48
Exp. 2 (G.H. prep. 508B)				
1. Saline injection				
A. Untreated	4	2.6±0.42		3.7±0.75
B. Growth hormone 1 mg. per 100 gm.	4	2.4±0.72	−0.2±0.83	3.1±0.42
2. Amino acid injection (12.9 mg. N per 100 gm.)				
A. Untreated	7	13.1±0.25		3.7±0.30
B. Growth hormone				
a. 0.1 mg. per 100 gm.	4	11.0±0.62	−2.1±0.57	3.4±0.51
b. 0.4 mg. per 100 gm.	5	9.6±0.73	−3.5±0.68	2.4±0.29
c. 1.0 mg. per 100 gm.	4	8.2±0.20	−4.9±0.37	3.3±0.46
3. Increase with amino acid injection over saline control series				
A. Untreated	11	+10.5±0.46		0.0±0.68
B. Growth hormone				
a. 0.1 mg. per 100 gm.	8	+ 8.6±0.75		+0.3±0.65
b. 0.4 mg. per 100 gm.	9	+ 7.2±1.0		−0.7±0.49
c. 1.0 mg. per 100 gm.	8	+ 5.8±0.74		+0.2±0.62

tion of the casein hydrolysate was reduced by about $\frac{1}{3}$ in the rats which had been given growth hormone in a dose of 1 mg. per 100 gm. body weight. The same dose of growth hormone had no detectable effect on the basal rate of urea production during this time. In the second and third hours after the injection of the amino acids, when urea formation had fallen to control levels, there was again no effect of the hormone observable.

When the dose of growth hormone was reduced to 0.4 or to 0.1 mg. per 100 gm., a depression in the rate of urea production after the administration of the hydrolysate was less, but still evident (−27 and −16 per cent respectively). A plot of the increase in blood urea against the logarithm of the dosage indicated a linear relationship with a significant slope ($b = -2.82 \pm 0.85$ mg. per cent per log. unit dosage), and no significant

departure from linearity with the 3 dosage groups employed. The index of precision ($\lambda = s/b$) was 0.43, indicating that the relationship possibly might be used for assay purposes, but with only a relatively low order of precision unless large numbers of animals were used.

If the urea in the blood is considered to represent the urea of the body water, the rates of urea production per unit body weight may be estimated for comparison with the amount of nitrogen injected. The results of such calculations (made on the assumption that urea is distributed in 63 per cent of the body weight) are presented in Figure 1. The difference between the amount of nitrogen injected and that converted to urea during the first hour may be considered to be nitrogen "retained." In this case in the three groups of animals given 0.1, 0.4, and 1.0 mg. of growth hormone, the in-

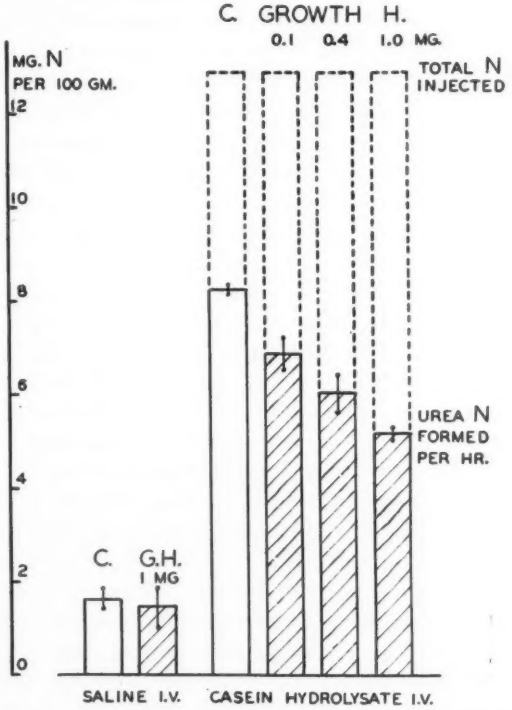

FIG. 1. The effect of growth hormone on the rate of urea formation and on the "retention" of nitrogen during the first hour after the injection of saline or of casein hydrolysate. The columns outlined in solid lines represent the rates of accumulation of urea nitrogen per 100 gm. body weight per hour (C = control values from untreated animals), and the broken lines the total amount of nitrogen injected and the amounts of nitrogen not metabolized to urea (i.e., "retained") during this time.

creases in nitrogen retention over that seen in the controls were 30 ± 10, 48 ± 11, and 67 ± 7 per cent respectively.

DISCUSSION

The observations reported earlier (Russell and Cappiello, 1949) indicated that injected amino acids were removed from the blood of the growth hormone treated animals at least as fast as in the control animals or perhaps more rapidly. The decrease in the rate of urea formation after growth hormone therefore may be presumed to result from increased retention of amino acids in the tissues. It seems likely that this retention represents increased synthesis of protein, although it cannot be certain at present that the "retained" amino acids are not held in the tissues in some non-protein form. In any case the fact that under these conditions no effect of growth hormone on the rate of formation of urea could be detected in the absence of administered amino acids would appear to indicate that the principal action of the hormone had been on the uptake of amino acids into the tissues, rather than on the catabolism of protein or of amino acids. This conclusion is in agreement with that of Friedberg and Greenberg (1948), who observed that the rate of uptake of methionine labeled with isotopic sulfur into skeletal muscle of rats and mice was accelerated in animals treated with growth hormone for several days.

From data obtained on the rates of excretion of isotopic nitrogen in fasting rats given small amounts of labeled glycine, Hoberman (1950) considered that growth hormone affected directly neither the synthesis nor the breakdown of body protein but instead decreased the rate of catabolism and excretion of amino acids; and he interpreted the effect of growth hormone on the rate of urea formation, described above, as in accord with this conclusion. In the three series of experiments carried out in hypophysectomized, adrenalectomized, or thyroidectomized rats, the rate of breakdown of body protein (determined in effect by the rate of dilution of isotope by non-isotopic nitrogen from the body)[3] was unaffected by growth hormone. In the same animals, the excretion of nitrogen was decreased noticeably in all instances. In the absence of change in the catabolism of protein, this retention of nitrogen then would have to be ascribed either to an increased rate of synthesis of protein, or to a commensurate increase in the quantity of non-protein amino nitrogen in the body. The latter change seems unlikely, since the average "nitrogen pool" size was not altered significantly by the hormone, and since the concentration of amino acids in the blood is diminished in similar circumstances (Li *et al.*, 1949;

[3] $M = E/\alpha$, where M = rate of protein breakdown ($= k_3P$ in Hoberman's notation), E = rate of excretion of nitrogen, and α = fraction of injected isotope excreted at "infinite" time.

Russell and Cappiello, 1949; Milman and Russell, 1950). The amount of free amino nitrogen in the tissues has been reported also to be decreased after treatment with anterior pituitary extracts (Shaffer and Lee, 1935). From calculations of the "rate constants" for synthesis of protein from the pool (fraction of labeled pool nitrogen removed per hour by means other than excretion), Hoberman could not find that growth hormone had a consistent effect upon synthesis; but from the rates of synthesis calculated in terms of mg. per 100 gm. per hour[4] (see Russell, 1951), and from the considerations outlined above, it appears that these experiments too would indicate an effect of growth hormone on the synthesis of protein.

Hoberman suggested that the growth hormone had a specific effect upon the catabolism of amino acids because the fraction of the "pool" nitrogen undergoing catabolism and excretion was decreased in the treated animals. If this were the sole process affected by growth hormone, an accumulation of non-protein nitrogen would be expected to result, but as noted above, this has not been seen. Moreover, the rate of urea formation by the liver in fasting animals is only a fraction of that found after the administration of amino acids (in either growth hormone treated or normal animals), so that limitation by growth hormone of the rates of deamination or urea formation *per se* is difficult to conceive. Rather, the reduction in excretion of nitrogen from the pool may be interpreted as a resultant of the diminution in net rate of protein loss from the body.

The smallest dose of growth hormone employed here, 0.1 mg. per 100 gm. per day, still produced a detectable reduction in the rate of urea formation after the administration of the protein hydrolysate. No attempt was made here to find the optimum time or route of administration of the hormone, so that it is quite possible that the minimum effective dose would be less than this amount. It may be noted that this dosage is not greatly in excess of the amount of purified growth hormone required daily to produce consistent increases in nitrogen retention in the hypophysectomized rat (Bennett, *et al.*, 1948), and is of about the same order as that seen to induce nitrogen retention and moderate gains in weight in the normal adult rat (Whitney, *et al.*, 1948). It is likely then that the "retention" of nitrogen observed here in acute experiments in the nephrectomized rat is a counterpart of the retention of nitrogen and growth which is observed when growth hormone is given in more physiological circumstances.

SUMMARY

The effect of highly purified growth hormone on the rate of accumulation of urea in the blood of nephrectomized rats was determined after the ad-

[4] $S = M - E = E(1-\alpha)/\alpha$ = rate of synthesis of body protein.

ministration of casein hydrolysate or of salt solution. The increase in blood urea during the first hour after the injection of the amino acids was reduced by about one-third in the animals given 1 mg. of growth hormone per 100 gm. body weight, and by a still detectable degree when the dose was reduced to 0.1 mg. per 100 gm. No effect of the larger dosage on the basal rate of urea formation was seen during the same time interval in animals not given the protein hydrolysate. These results were interpreted to indicate an effect of the growth hormone on the retention of amino acids in in the tissues rather than on the catabolism of the amino acids.

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EFFECT OF HYPOPHYSECTOMY AND ADRENALECTOMY ON SUCCINOXIDASE ACTIVITY IN THE LIVERS OF ALLOXAN DIABETIC RATS

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MEYER and McShan (1950) have reported that the succinoxidase activity in the livers of lactating alloxan diabetic rats and alloxan diabetic male rats is elevated above that of nondiabetic controls within five days after administration of alloxan. Houssay (1936) has reported that hypophysectomy ameliorates pancreatic diabetes in many animals; Long, Katzin and Fry (1940) have reported that adrenalectomy also ameliorates pancreatic diabetes in the rat. In this paper the effect of adrenalectomy and hypophysectomy on succinoxidase in the livers of alloxan diabetic rats has been determined. It has been found that these operations reduce the elevated succinoxidase of the alloxan diabetic rat to that of the corresponding nondiabetic control.

METHOD

Male Holtzman strain rats weighing 200 to 300 gms. were used. A group of them was made diabetic with alloxan using the method of Kass and Waisbren (1945). After being starved for two days the rats were injected subcutaneously with an alloxan solution containing sixteen mg. of alloxan per ml. The dose used was 1 ml. per 100 gm. body weight. Five days after the administration of alloxan the rats were tested to see which were diabetic by determining the glucose level in the blood five hours after a meal. The rats were starved for twelve hours after which a meal of five grams of pelleted stock diet was given to each rat. The food was eaten in one-half to three quarters of an hour. Five hours later the glucose level in the blood from the tail of unanesthetized animals was determined by the method of Somogyi (1945) and Nelson (1944). Rats having blood sugar levels over 350 mg. per cent were considered diabetic. A majority of the rats had blood sugar levels over 500 mg. per cent. Six days after the administration of alloxan, eight diabetic and eight normal rats were adrenalectomized through the dorsal approach, and the next day eight diabetics and eight normal rats were hypophysectomized through the parapharyngeal approach. Each animal was kept in a separate cage with a wire mesh bottom.

The rats were maintained on a stock diet up to the start of the experiment. They were started on a bread and milk diet on the day the rats were adrenalectomized. All adrenalectomized rats were given 1% saline as drinking water. The diet was chosen because of our concern over the maintenance of the adrenalectomized rats, which die rapidly when fed our stock diet, but do very well on bread and milk. All except the hypophysectomized

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rats were presented daily with one and one-half to two fresh slices of white bread soaked with fifteen ml. milk. A slice of bread weighed approximately 25 gm. The hypophysectomized rats were given three-fourths of a slice soaked with fifteen ml. of milk. A record was kept of a rough estimate of food left over and discarded each day, and of water intake. A summary of daily food and water intake together with the average weight gain of the animals in each group is given in Table 1.

It was found that all diabetics drank significantly more water than their nondiabetic controls, though hypophysectomy decreased water intake to about half that of the unoperated diabetics. The unoperated diabetics ate all their food, the normals did not. It is possible that the diabetics would have eaten even more if more food were presented.

TABLE 1. AVERAGE WEIGHT GAIN IN 2 WEEKS, DAILY BREAD AND WATER INTAKE OF EXPERIMENTAL RATS

	Normal	Adrenal-ectomized	Hypophy-sectomized	Alloxan Diabetic	Adrenal-ectomized Diabetic	Hypophy-sectomized Diabetic
Number of rats	7	8	6	5	7	6
Bread gm.	25 to 31	25 to 31	12 to 19	40 to 50	25 to 31	12 to 19
Water ml.	5 to 30	5 to 40	30	125 to 200	125 to 160	60 to 125
Wt. gain gms.	44 ± 7*	41 ± 19*	-11 ± 11*	1 ± 24*	-4 ± 19*	-17 ± 14

* Mean ± Standard deviation.

No tests were made for completeness of adrenalectomy. Tests had shown absence of significant amounts of ectopic adrenal tissue in general in this strain of rat since adrenalectomized rats maintained on bread and milk and 1% saline for two weeks and then placed on stock diet and water died within a week after change of diet.

Only visual tests were made for completeness of hypophysectomy. No hypophysectomized rats gained weight during the experimental period and all had atrophied gonads. Hypophysectomized nondiabetics were inspected in the region of the sella turcica to insure absence of remaining pituitary tissue.

Between 14 and 19 days after hypophysectomy or adrenalectomy a blood[1] sample was taken by cardiac puncture and the unfasted rats were then killed by decapitation, bled, and samples of liver were removed from the median lobe, weighed on a torsion balance and homogenized at 0°C. in cold distilled water to make a five per cent homogenate. Stoppered tubes of homogenates were kept in a beaker of ice in the refrigerator until used about six hours later.

Succinoxidase was determined by the method of Schneider and Potter (1943) using cytochrome-C obtained from the Sigma Chemical Company.

Nitrogen was determined by direct Nesslerization using a procedure similar to that in Umbreit, Burris and Stauffer (1945) modified by using six additions of 30% H_2O_2 to get a more complete digestion (Miller and Miller 1948).

RESULTS

Data on nitrogen content, and succinoxidase activity on the basis of fresh weight and nitrogen content are shown in Table 2.

The livers of alloxan diabetic rats contain about 30 per cent more nitrogen per unit fresh weight than do normal livers. Adrenalectomized or hypophysectomized diabetic rats have a liver nitrogen content equal to

[1] Data obtained from this sample will be reported in another paper.

that of adrenalectomized or hypophysectomized controls, both of which have liver nitrogen contents elevated above that in normal rats. Livers of three normal and three alloxan diabetic rats have been examined for water content and no difference has been found since the livers of both groups had an average of 68 per cent water. It may be that some of the difference in nitrogen in the liver is due to loss of glycogen in the diabetic, but this will probably not account for the entire difference.

Succinoxidase activity of diabetic livers is about 70% higher than in corresponding normal livers on a fresh weight basis. Adrenalectomy or hypophysectomy of the diabetic rats caused the succinoxidase activity of their livers to decrease to about the activity of the corresponding adrenalectomized or hypophysectomized controls. Adrenalectomized rats have

TABLE 2. SUCCINOXIDASE DETERMINATIONS ON LIVERS OF ADRENALECTOMIZED DIABETICS, HYPOPHYSECTOMIZED DIABETICS AND CONTROLS

Treatment	μgN 5 mg. fresh wt.	P†	Cu. mm. O_2 10 min.×20 mg. fresh liver	P	Cu. mm. O_2 Hour×mg. N	P
Normal	142±3* (7)		66±2 (7)		691±13 (7)	
Adrenalectomized	154±5 (8)	A,N. .1	72±3 (8)	A,N .2	698±14 (8)	A,N .8
Hypophysectomized	167±3 (6)	H,N. .01	76±3 (6)	H,N .02	686±21 (6)	H,N .9
Diabetic	184±7 (5)	D,N .01	111±7 (5)	D,N .01	908±27 (5)	D,N .01
Adrenalectomized Diabetic	156±2 (6)	AD,A .9	76±3 (7)	AD,A .5	714±25 (6)	AD,A .6
Hypophysectomized Diabetic	162±3 (6)	HD,H .3	81±5 (6)	HD,H .5	745±29 (6)	HD,H .2

* Mean ± standard error of the mean.
† Probability that difference is due to accident for indicated groups being compared.
Figures in parentheses are the number of rats used to determine the mean.
N—Normal
D—Diabetic
A—Adrenalectomized
H—Hypophysectomized

essentially normal succinoxidase activity while hypophysectomized rats have slightly elevated activity.

If the data are analyzed on the basis of oxygen produced per mg. liver nitrogen a slightly different picture is obtained. Diabetics are elevated above normal only by about 25 per cent.[2] Adrenalectomy and hypophysectomy reduce the elevated activity in the diabetic livers almost to normal. Adrenalectomized and hypophysectomized rats have normal activity.

DISCUSSION

A few data are available in the literature on liver nitrogen of adrenalectomized (Levin, 1945) and hypophysectomized (Vail and Kochakian,

[2] The figures on liver nitrogen reported in this paper should not be taken to suggest that the elevation in succinoxidase in alloxan diabetics reported by Meyer and McShan (19) is a trivial effect closely related to elevation in liver nitrogen. Unreported data obtained using normals and alloxan diabetics maintained on a commercial pelleted diet supplemented with 10 per cent liver powder indicate that in this case the elevation in liver nitrogen content of the diabetics is not over 15% that of the normal while the succinoxidase activity is still grossly elevated.

1947) rats. These workers find only small differences between normal and operated animals while we find elevation in the operated animals significant for the hypophysectomized rat. However, their animals were starved 18 hours or more; ours were fed a different diet until sacrifice.

Determinations of succinoxidase activity in rat livers have been made by many investigators. Succinoxidase activity has been reported to depend upon diet (Potter *et al.*, 1950; Tipton 1950), thyroid activity (Tipton *et al.*, 1946), adrenal activity (Tipton, Leath *et al.*, 1946), an intact pancreas (Meyer and McShan, 1950), animal age (Potter *et al.*, 1945), and other variables. It has been suggested that adrenalectomy causes a slight decrease (Tipton, Leath *et al.*, 1946), but our data using a different diet show no significant difference in succinoxidase activity in the livers of adrenalectomized rats and normal controls, Table 2. Hypophysectomized rats show an elevated succinoxidase activity which is proportional to the changed nitrogen content of the fresh liver. The significance of the elevated succinoxidase in the livers of the alloxan diabetic rat is unknown at present. Adrenalectomy or hypophysectomy returns the elevated activity to the adrenalectomized or hypophysectomized level; this suggests that the elevation in succinoxidase produced when a rat is made diabetic with alloxan requires the presence of pituitary and the adrenal glands.

SUMMARY

Normal, alloxan diabetic, adrenalectomized, hypophysectomized, adrenalectomized diabetic and hypophysectomized diabetic rats were maintained on a bread and milk diet. All adrenalectomized rats were given 1% saline as drinking water. Livers were examined for nitrogen content and succinoxidase activity.

The liver nitrogen of the unfasted alloxan diabetic rat was elevated 30 per cent above normal, though the per cent water was normal. Liver succinoxidase activity was 70 per cent above normal per unit fresh weight, but only 25 per cent above normal per mg. nitrogen. Per cent liver nitrogen and liver succinoxidase were brought down to that of the corresponding nondiabetic controls by adrenalectomy or hypophysectomy.

The livers of normal and adrenalectomized rats have the same nitrogen content and succinoxidase activity per gm. fresh weight. The livers of our hypophysectomized rats have slightly elevated nitrogen content and an elevated succinoxidase activity which is proportional to the elevation in liver nitrogen.

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HISTOCHEMICAL DEMONSTRATION OF KETOSTEROIDS IN VIRILIZING TUMORS OF THE ADRENAL CORTEX[1]

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A METHOD for the histochemical demonstration of active carbonyl groups of lipoid and non-lipoid material has been described by us (Ashbel and Seligman, 1949; Seligman and Ashbel, 1949; Seligman, Friedman and Herz, 1949). After formalin fixation of the tissue, it was possible to demonstrate active carbonyl groups in some of the lipoid of various endocrine glands such as adrenal cortex, corpus luteum, testicular interstitial cells, and syncytial trophoblasts of placenta (Ashbel and Seligman, 1949). Reasons for considering the material thus demonstrated to be ketosteroid have been discussed (Ashbel and Seligman, 1949) and more evidence for this claim appears below. Material, which is found in the white matter of the central nervous system, in the myelin sheath of peripheral nerve, and in other tissues, is poorly soluble in lipoid solvents, contains carbonyl groups after fixation in formalin (Seligman and Ashbel, 1951), and is considered to be similar to proteolipid isolated from the white matter of brain by Folch and Lees (1950).

This communication presents data on the correlation of the clinical with the histochemical findings as demonstrated by this method in virilizing tumors of the adrenal cortex. An experimentally produced adrenal cortical carcinoma was also studied.

METHOD

Frozen sections, cut 10–20 microns in thickness from tumors of the adrenal cortex, which had been fixed in neutral 10% formalin for a few days to as long as 11 years, were

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[2] On leave of absence from the Hebrew University, Jerusalem, Israel. Research fellow in Surgery, Harvard Medical School. This fellowship was made possible by a grant from the Jane Coffin Child Memorial Fund for Medical Research, Yale University, 1949, and by an institutional grant to Harvard University from the American Cancer Society, 1950.

attached to glass slides by allowing them to dry for 5–10 minutes. They were then washed for 2 hours in several changes of water to remove the formalin. Sections from each block of tumor tissue were stained for fat with sudan IV for a comparison with sections for carbonyl groups. In order to determine whether carbonyl containing material was lipoidal in nature, other sections were dehydrated with alcohol, immersed in alcohol-xylol or chloroform for a few hours or in acetone for 24 hours, and then hydrated with dilute alcohol and washed with water, prior to staining for carbonyl groups. Some sections, already stained for carbonyl groups, were dehydrated and extracted with the solvents mentioned above. Still other sections were stained with hematoxylin-eosin.

Normal adrenal cortex was extensively studied with lipoidal solvents and reactions aimed to distinguish aldehydic from ketonic carbonyl. The tumors were subjected to some of the procedures which proved the ketonic character of the lipoidal carbonyl groups.

The test[3] for active carbonyl groups was conducted as previously described (Ashbel and Seligman, 1949 and Seligman and Ashbel, 1951) with the reagents, 3-hydroxy-2-naphthoic acid hydrazide and tetrazotized diorthoanisidine. The stained sections were washed in several changes of water and finally, to avoid bubbles, with water from which dissolved gases had been allowed to escape by standing at room temperature. The sections were mounted in glycerogel. Black and white photomicrographs of sections stained for ketosteroids were taken through a red filter and sections stained with sudan IV were photographed through a green filter. A light blue filter was used for some of the colored photographs.[4]

RESULTS

Normal adrenal cortex

Fresh adrenal glands from rats, rabbits, dogs, and human subjects (Figs. 1 and 2) were used in the following study:

Solvents: In order to demonstrate the lipoidal nature of the stained material in the adrenal cortex, sections were cut from tissue fixed in neutral 10% formalin for a few hours, months or years, washed with distilled water, dehydrated by immersion in graded concentrations of alcohol for ½ hour each, and finally incubated at room temperature for 24 hours in various solvents. These were absolute ethanol, acetone, pyridine, chloroform, ether, xylol, and alcohol-xylol. The sections were then hydrated and stained for carbonyl groups. The reaction was completely blocked by all these solvents which had removed the reacting material.

When sections were stained for carbonyl groups and then dehydrated and incubated in the same solvents, most of the blue pigment was removed. The endothelium of sinusoids and some of the erythrocytes were still stained, suggesting the weakly lipoidal properties of the stainable material in these sites (see earlier publications of Ashbel and Seligman, 1949 and Seligman and Ashbel, 1951).

[3] All the reagents for this reaction may be purchased from Dajac Laboratories, Monomer-Polymer, Inc., 511 Lancaster Ave., Leominster, Mass.

[4] Photomicrographs by Mr. Leo Goodman.

Fresh tissues: Fresh frozen sections of adrenal cortex failed to react for carbonyl groups after exposure to 3-hydroxy-2-naphthoic acid hydrazide for 2 hours. Prior immersion of such sections in 0.5% hydrochloric acid for 10 minutes or 1 hour did not produce a positive reaction for carbonyl groups. When sections were first treated with 10% mercuric chloride for 5 minutes or 1 hour a diffuse blue color was produced in the reticularis. Nothing resembling the reaction in formalin-fixed tissue was seen with these procedures, which would be expected to liberate plasmal from plasmalogen.

Evidence for the ketonic character of the carbonyl group in formalin-fixed adrenal cortex

No intensification or alteration of the stained pattern in formalin-fixed sections of adrenal cortex was produced by treatment of the sections with 10% mercuric chloride for 10 minutes prior to staining.

No reaction for aldehyde was produced by reaction of formalin-fixed adrenal cortex with phenyl hydrazine followed by treatment in the cold with tetrazotized diorthoanisidine in aqueous solutions of pyridine or pyridinium acetate. This reaction produces colored formazans (Ashbel and Seligman, 1949; Rutenburg, Gofstein and Seligman, 1950; Seligman, Gofstein and Rutenburg, 1949; Seligman and Ashbel, 1951) with aldehydes (see formulation in Fig. 15 of previous publication Seligman and

PLATE I

FIG. 1. Normal adrenal gland. Ketosteroid reaction was positive in the lipoid laden cells of the three zones of the adrenal cortex. The reaction was most intense in the fasciculata and reticularis. The medulla (right) had no significant reaction. This section had been incubated with sulfanilic acid at pH 3–5 at 37°C. for 18 hours to form Schiff's base with aldehydic material, prior to staining for ketosteroid. No decrease in the usual degree of staining for ketosteroid was observed. ×110.

FIG. 2. Same section as Figure 1. Ketosteroid reaction in the lipoid droplets of the cytoplasm of cells in the outer fasciculata or glomerulosa is shown. ×1300.

FIG. 3. Case 1, adrenal cortical carcinoma. Ketosteroid reaction was positive in the lipoid droplets of tumor cells. ×400.

FIG. 4. Case 1. Sudan IV stained lipoid droplets in tumor cells. Marked variation in number and size of lipoid droplets are shown. ×400.

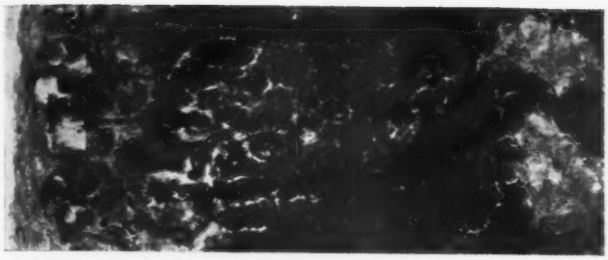
FIG. 5. Adrenal cortical carcinoma of C_3H mouse, first generation transplant. Ketosteroid reaction was positive in the lipoid of the cytoplasm of tumor cells. ×400.

FIG. 6. Case 2, malignant tumor of persistent fetal cortex of the adrenal. Ketosteroid reaction was positive (blue color) in the lipoid of isolated cells and in the lipoid between vacuoles of the bizarre foam cells. ×400.

FIG. 7. Case 2. Sudan IV stained the lipoid in isolated cells and the lipoid between vacuoles of the bizarre foam cells. ×400.

FIG. 8. Case 5, adrenal cortical adenoma. Reaction for ketosteroid occurred in the uniformly sized droplets of tumor cells. ×400.

FIG. 9. Case 5. Sudan IV stained the uniformly sized droplets of lipoid. Their number in the cytoplasm of tumor cells varied. Occasional large fat droplets were stained. ×400.



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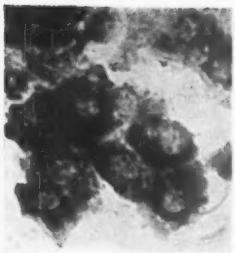

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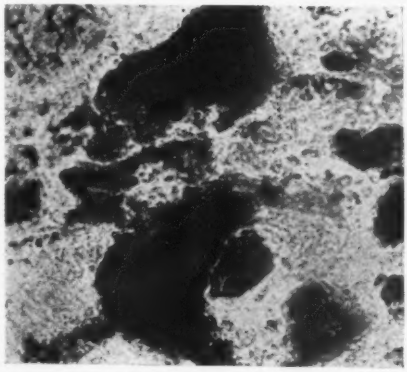

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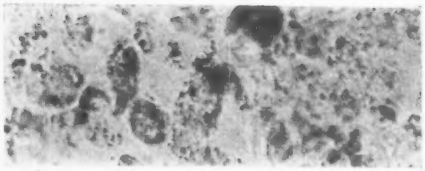

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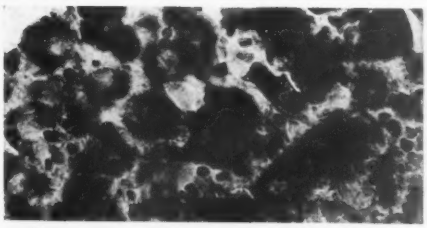

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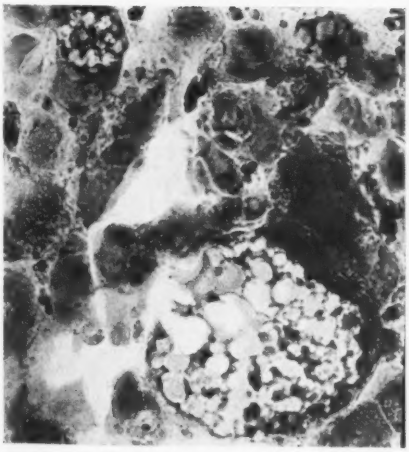

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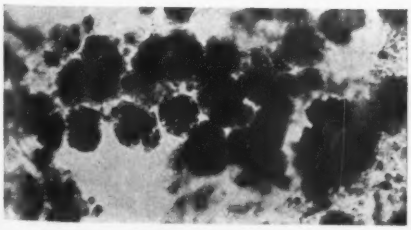

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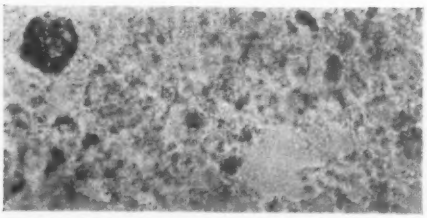

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Ashbel, 1951). The reaction is specific for aldehydes, since, in the absence of replaceable hydrogen, coupling with tetrazotized diorthoanisidine cannot occur on hydrazones produced from ketones.

The Angeli-Rimini test for aldehydes, which is performed with benzenesulfohydroxamic acid (Karrer, 1938 and Seligman and Ashbel, 1951) in the presence of iron salts, failed to give the red color of the alkyl hydroxamic acid which forms only with aldehydes.

Further evidence that aldehyde was not present was provided by the fact that prior incubation at pH 3–5 for 18 hours at 37° in sulfanilic acid or aniline (1.0 mg./cc.), which is claimed to react specifically under these conditions with aldehydes to produce Schiff's base (Oster and Mulinos, 1944), did not block at all the reaction for carbonyl groups in nervous tissue (Seligman and Ashbel), in normal adrenal cortex (Figs. 1 and 2), or in all the tumors described below.

Reversal of the reaction of ketones with the hydrazide

An excess of the sodium salt of *o*-sulfobenzaldehyde (5%) was able to reverse the reaction of ketones with 3-hydroxy-2-naphthoic acid hydrazide rapidly and extensively, although not completely at room temperature. The reaction with aldehydes was reversed at a very much slower rate at room temperature (see Figure 16 of earlier publication (Seligman and Ashbel)). Sections of formalin-fixed adrenal cortex (and cortical tumors described below) which had been treated with the hydrazide, were incubated at room temperature in a solution of the sodium salt of *o*-sulfobenzaldehyde (5%) for several hours or for 3 days, and washed with a solution of sodium bicarbonate for a few minutes and with several changes of water for 24 hours in order to remove the water soluble hydrazone produced with the *o*-sulfobenzaldehyde. The tissue sections were then treated with the diazonium salt and the intensity of the stain was compared with controls. The sections were strikingly paler than controls or nearly colorless, suggesting that the carbonyl group was ketonic in nature.

Since pyruvic acid has a similarly reactive carbonyl group, it was also able to reverse the reaction with the hydrazide in sections of adrenal cortex.

When sections were impregnated with a long chain aldehyde by immersion in a solution of stearal[5] in 60% alcohol for 2 hours, intense staining was noted in lipoid droplets when the reaction for carbonyl groups was performed. However, this reaction was not reversed by incubation at room temperature for a few hours in a solution of *o*-sulfobenzaldehyde (5%). This showed that the test reliably distinguished ketonic from aldehydic

[5] Provided through the courtesy of Dr. S. J. Tannhauser, New England Medical Center, Boston.

carbonyl groups. Pyruvic acid in large excess reversed this reaction almost as rapidly as in sections which were not treated with plasmal. Therefore, pyruvic acid reversal cannot be used conveniently to distinguish ketonic from aldehydic carbonyl groups.

Some hydrolysis of the hydrazone (produced in sections of adrenal cortex with the hydrazide) occurred when sections were washed in water for 5 days. However, hydrolysis as indicated by slightly paler sections than controls, was much less than had been observed with nervous tissue (Seligman and Ashbel, 1951).

TABLE 1. ADRENAL TUMORS OF HUMAN SUBJECTS CAUSING VIRILISM

Case No.	Experiment No.	Sex	Age Years	Diagnosis	Period of fixation in formalin Years	Size of droplets which reacted for ketosteroid	Urinary output of 17-ketosteroid at time of operation Mg. in 24 hours	Weight of tumor Grams
1	156	F	3	Adrenal Cortical Carcinoma	4	large	385–485	1500
2	281	F	3¾	Malignant tumor of of persistent fetal cortex of the adrenal	11	large	122–220	110
3	288	M	6½	Adrenal Cortical Carcinoma	0.5	small	6.6	14
4	189	F	29	Adrenal Cortical Carcinoma	3	small	271	150
5	589	F	40	Adrenal Cortical Adenoma	0.05	medium	115	2000

VIRILIZING TUMORS OF THE ADRENAL CORTEX (TABLE 1)

Case 1:

156 (S-45-2648) B.I.H.: A 3 year old girl was admitted to this hospital in 1945 with a chief complaint of abdominal pain of two months' duration. Her development had been precocious. She walked and talked at 6 months' of age. She began to grow long black pubic hair and axillary hair at one year of age, and she was the size of a 7 year old girl with abnormal muscular development at 3 years. Her blood pressure was 180/120 mm. Hg. There was marked enlargement of the clitoris. Her abdomen was distended with a large mass in the left upper quadrant extending to the midline and to the flanks and below the umbilicus anteriorly. The mass was non-tender. By roentgenogram the development of the bones of the extremities corresponded to that of 8 years. The stomach was displaced to the right by a large mass in the left upper quadrant and there was marked displacement of the left kidney downward, with deformity of the upper calyx.

Her urine during 24 hours contained 385–485 mgs. of 17-ketosteroids and 1.3 mgs. of 17-hydroxy-corticosteroids.

At operation, a large left supra-renal tumor weighing 1500 gms. was

found. The tumor was soft and hemorrhagic. In the process of removal of the tumor and kidney, the thin capsule was ruptured. The pathologic diagnosis was adrenal cortical carcinoma with invasion of kidney. The urinary 17-ketosteroids dropped to 4 mgs. post-operatively. The patient returned a year later presenting a picture of advanced malignant disease. The precocious body development and secondary sexual characteristics were even more marked than on the former admission. The abdomen was protruberant, tense and tender. The output of 17-ketosteroid in 24 hours was 350–800 mgs. The patient expired 2 months later. At autopsy, nearly all the viscera were involved with carcinoma.

Microscopic examination

Areas of tumor which were free of obvious necrosis and hemorrhage consisted of cords and clusters of undifferentiated cells. The cells were large, polyhedral in shape and contained acidophilic, occasionally vacuolated cytoplasm and pleomorphic densely basophilic or vesicular nuclei (Fig. 10). Nucleoli were frequently present. Mitotic figures were noted. A capsule or pseudocapsule was seen at the periphery of certain portions of the tumor which appeared to be invaded by nests of tumor cells.

Ketosteroid reaction

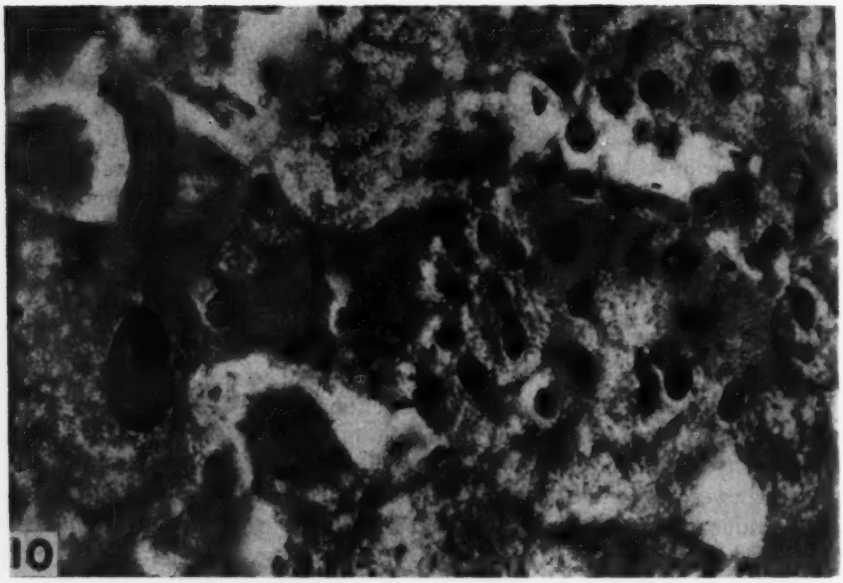
The cytoplasm of individual cells and of clusters of tumor cells at the periphery of the extensively necrotic tumor contained fine and large droplets of deeply blue-stained material (Fig. 3). The reaction was blocked by prior extraction with acetone and sections stained with sudan IV demonstrated the same lipid material (Fig. 4).

Case 2:

281 (S-38-178) Children's Hospital: A $3\frac{3}{4}$ year old girl was admitted to the hospital because of abnormal development. She sat up at 6 months, walked at 12 months and talked at 14 months. She was large and strong for her age. Her vulva had enlarged and pubic hair appeared during the first year. The clitoris grew to 1.5 inches in length during the second year and protruded from the labia (pseudohermaphrodite). Her voice was deep. The blood pressure was 116/50 mm. Hg. Roentgenograms revealed a bone development of 9–10 years. Assay of her urine for 17-ketosteroid was 122–220 mgs. in 24 hours. An adrenal tumor which weighed 110 grams was removed. The pathologic diagnosis was malignant tumor of persistent fetal cortex of the adrenal.

Microscopic examination

The tumor cells varied from small undifferentiated cells to large, ir-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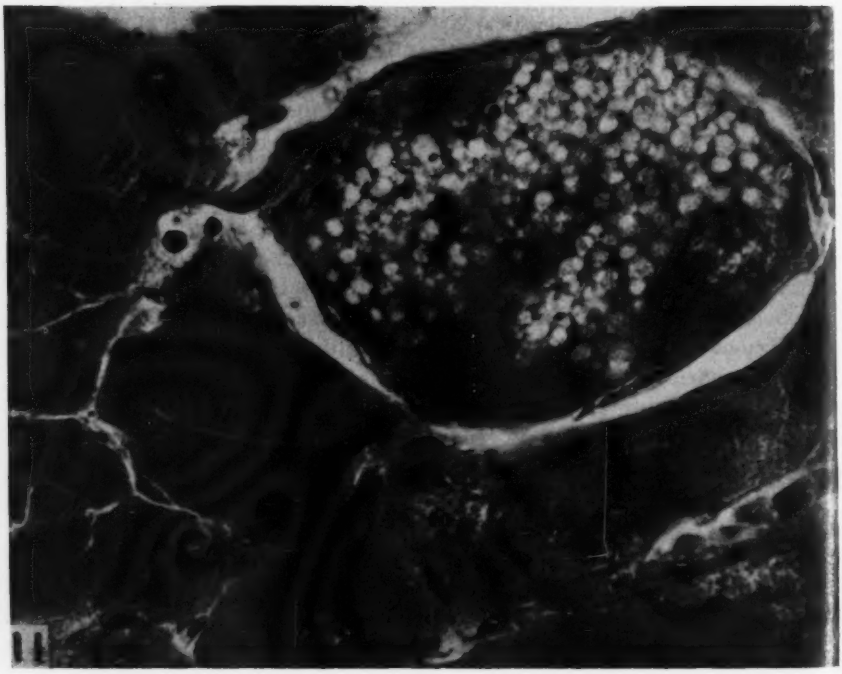
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regularly shaped giant cells containing many pleomorphic nuclei. Some nuclei were small round and hyperchromatic, while others were large and vesicular. Some of the cells contained as many as 5 or 6 nuclei. Vacuoles were present in variable number and size in the cytoplasm of some cells. In some instances the vacuoles were large and multiple and occupied large areas of the cytoplasm (Fig. 11). Mitoses were not seen. The stroma was scant and formed a reticular network. There were patches of necrosis.

Ketosteroid reaction

The cytoplasm of the tumor cells took a reddish-purple stain and most of these cells were lightly stained for fat with Sudan IV. Other scattered cells contained fat droplets which reacted for ketosteroid. Rare foam cells with many large vacuoles stained a deep blue in irregular cytoplasmic areas between the vacuoles (Fig. 6). The same part of these bizarre cells stained for fat with sudan IV (Fig. 7). Pre-treatment of the sections with alcohol-xylol for 2 days blocked only the blue-stained fat and not the diffuse reddish purple areas. Therefore, ketosteroid was demonstrated only in the lipoid droplets of some tumor cells and in the lipoid of scattered, highly vacuolated bizarre cells.

Case 3:

288 (49-4802) Massachusetts General Hospital: A $6\frac{1}{2}$ year old boy was admitted to the hospital because of rapid gain of weight, development of pubic hair and enlargement of the penis during the preceding 6 months. His penis was 3 inches long and 0.5 inch in diameter. The blood pressure was 130/100 mm. Hg. The bone age was 7 years. A 24 hour output of urine contained 6.6 mgs. of 17-ketosteroid and 0.2 mg. of 17-hydroxy-corticosteroids. At operation, a globular, encapsulated tumor, weighing 14 gms., was found in the adrenal gland. The pathologic diagnosis was adrenal cortical carcinoma.

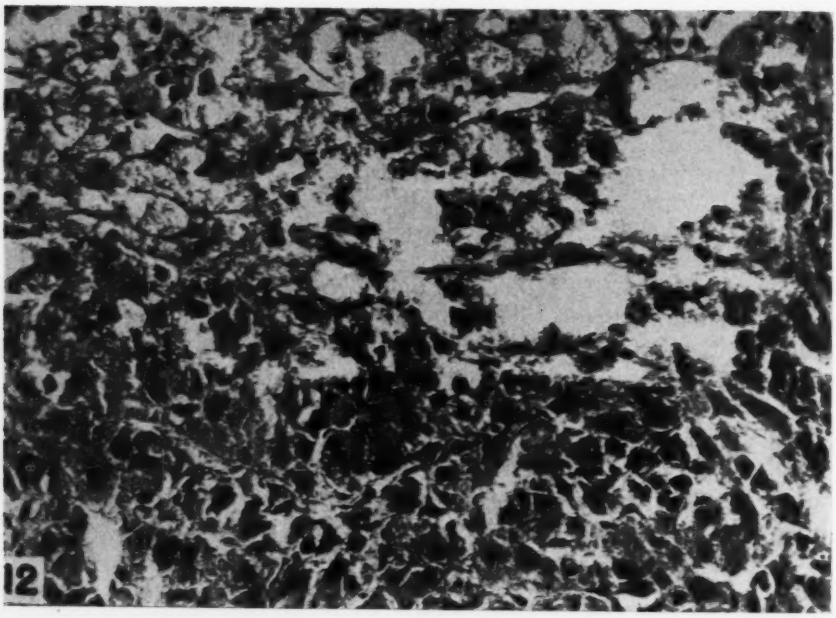
Microscopic examination

The tumor cells were undifferentiated. The cells formed sheets and varied in appearance in different parts of the tumor. In one portion the cells were large, polygonal in shape with abundant acidophilic cytoplasm (Fig. 12). The nuclei were pleomorphic and vesicular and contained clumps of

PLATE II

FIG. 10. Case 1, adrenal cortical carcinoma. Polyhedral cells, many with vacuolated cytoplasm and pleomorphic, densely basophilic nuclei. Hematoxylin-eosin stain. ×800.

FIG. 11. Case 2, malignant tumor of persistent fetal cortex of the adrenal. Variation in size of cells, multinucleated cells and a large vacuolated bizarre foam cell. Cell nuclei exhibit pleomorphism. Hematoxylin-eosin stain. ×800.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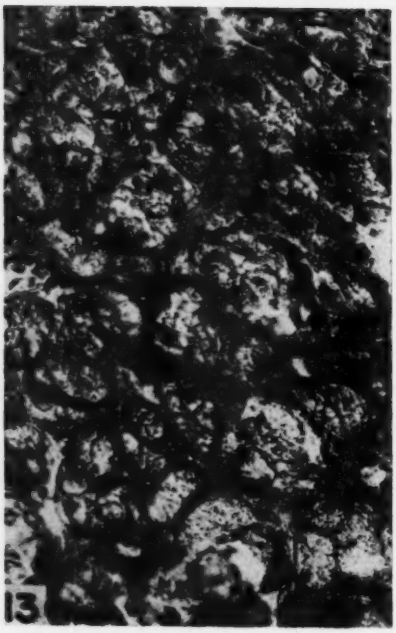
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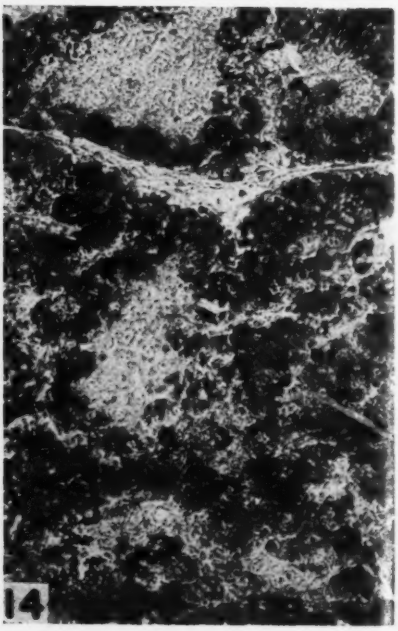
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chromatin and nucleoli. Occasionally large multinucleated cells were noted. In other sections of the tumor the cytoplasm of the cells became extensively vacuolated with lipoid droplets and some cells were almost completely replaced by large conglomerate collections of fat. The nuclei of these cells were eccentrically placed and intensely basophilic (Fig. 12). Mitotic figures were not seen. At the periphery, the tumor was undergoing fibrosis.

Ketosteroid reaction

Large areas of tumor contained cells whose cytoplasm contained small blue-stained droplets (Fig. 13). Tumor cells with little lipoid were stained weakly. The reaction was blocked by prior extraction with alcohol-xylol. The same cytoplasmic fat was stained with sudan IV in viable cells at the periphery of necrotic areas (Fig. 14).

Case 4:

189 (1-492949) Mayo Clinic: A 29 year old woman was admitted to the hospital in 1946 with a chief complaint of having developed abdominal pain in the right upper quadrant while driving a tractor, and a history of hirsutism for 10 years for which she had shaved daily. Her libido and the development of her muscles had increased. Her blood pressure was 236/180 mm. Hg. The 24 hour output of 17-ketosteroid in her urine was 271 mgms. A right adrenal cortical tumor, weighing 150 gms. was removed. The pathologic diagnosis was adrenal cortical carcinoma. Postoperatively the urinary 17-ketosteroid dropped to 5.8 mgs. and 2.5 mgs. All symptoms except hirsutism regressed. She appeared to be in good health and to have reverted to complete femininity on follow-up 2 years later.

Microscopic examination

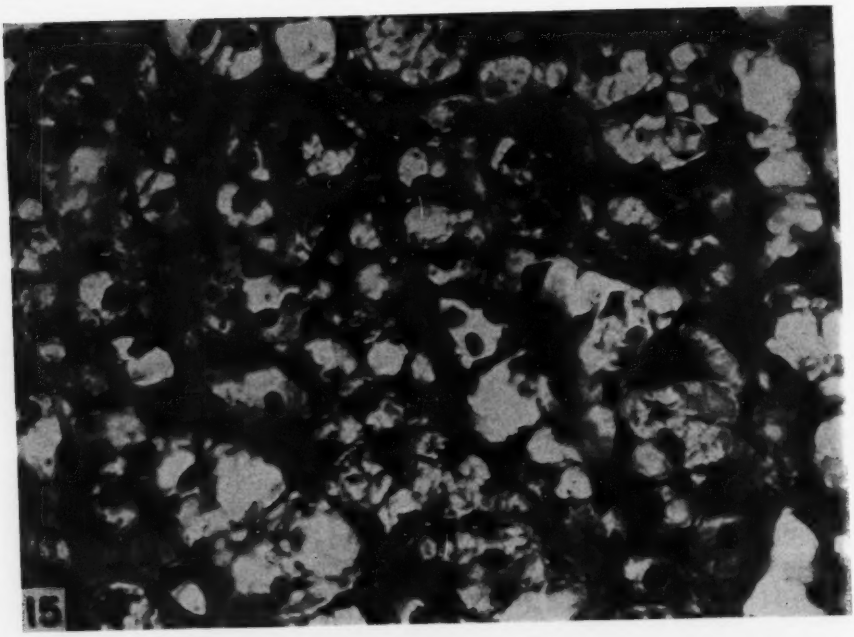
The tumor consisted of sheets of undifferentiated cells with large pleomorphic, vesicular, basophilic nuclei which contained one or more nucleoli. Mitotic figures were infrequent. In some areas, the cytoplasm was extensively vacuolated. In some instances the cytoplasm was almost completely replaced by these vacuoles with eccentrically placed nuclei (Fig. 15). In

PLATE III

FIG. 12. Case 3, adrenal cortical carcinoma. Pleomorphic, polygonal cells (below) and lipoid laden cells with eccentrically placed, intensely basophilic nuclei (above). Hematoxylin-eosin stain. ×400.

FIG. 13. Case 3. Ketosteroid reaction was positive in the fine droplets of lipoid in the cytoplasm of tumor cells. ×400.

FIG. 14. Case 3. Sudan IV stained the fine lipoid droplets in the cytoplasm of tumor cells. Necrotic cells which did not contain lipoid may be seen in the center of the clusters. ×400.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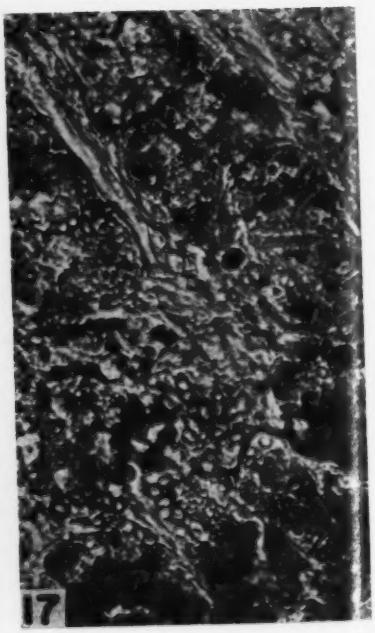
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other areas the cytoplasm was uniform, with little or no vacuolization. These cells appeared to fuse with surrounding cells to form a symplasmatic arrangement. The tumor was traversed by numerous blood vessels with thick or thin walls. There was congestion and in some areas extravasation of blood. Areas of extensive necrosis were also present.

Ketosteroid reaction

The cytoplasm of most of the tumor cells contained fine, light blue or purplish granules (Fig. 16). With sudan IV the same fine, light stippling was demonstrated in the cytoplasm of most of the cells (Fig. 17). Tumor cells in some areas contained large droplets of lipoid which also reacted for ketosteroid. This reaction in the cytoplasm was blocked by treatment of the sections for 24 hours with ethanol.

Case 5:

589 (50-4554) Allerton Hospital: A 40 year old woman was admitted to the hospital in 1950 with complaints of amenorrhea, hirsutism, abdominal pain and deepening of the voice. Examination revealed a mass the size of a grapefruit in the right upper quadrant, and a clitoris enlarged to five times normal size. The blood pressure was 110/60 mm. Hg. By roentgenogram the right kidney was displaced medially and the hepatic flexure of the colon was displaced inferiorly. The 24 hour output of urinary 17-ketosteroid was 115 mg. At operation, a large, encapsulated tumor of the adrenal gland, densely adherent to the right kidney but not invading the kidney was removed together with the kidney. The tumor which weighed about 2000 grams, was extensively necrotic. The pathologic diagnosis was adrenal cortical adenoma. Post-operatively the urinary output of 17-ketosteroids was 2.3 mgs. in 24 hours. The patient's facial appearance became more feminine due to a decrease in hirsutism and acne and her voice improved.

Microscopic examination

There was very extensive necrosis of the tumor. Viable tumor cells were clustered around blood vessels. The cells were uniform in pattern

PLATE IV

FIG. 15. Case 4, adrenal cortical carcinoma. An area of the tumor with cells having an extensively vacuolated cytoplasm is shown. Cell nuclei are hyperchromatic, pleomorphic and eccentrically placed. Hematoxylin-eosin stain. ×800.

FIG. 16. Case 4. Ketosteroid reaction was positive in the fine lipoid droplets as light blue or purplish granules. Erythrocytes reacted for carbonyl groups but this reaction was not blocked by prior extraction with ethanol. ×800.

FIG. 17. Case 4. Sudan IV stained lipoid droplets in tumor cells. Cells containing fine droplets and cells with large droplets are shown. ×800.

PLATE V

FIG. 18. Case 5, adrenal cortical adenoma. Cords of uniform cells are clustered around blood vessels. Hematoxylin-eosin stain. ×400.

and were comprised of cords and nests, 2–3 cells in width, in irregular patterns separated only by vascular strands of connective tissue (Fig. 18). The cytoplasm of the cells was granular and the nuclei were regular and deeply basophilic. Nucleoli were not prominent. Giant cells, multinucleated cells or vesiculation were not seen. Mitotic figures were not seen. There was no invasion of the capsule or of the blood vessels. In spite of evidence

of the rapid growth of this tumor, criteria of malignancy were not found in any of the many sections examined.

Ketosteroid reaction

Many of the tumor cells clustered around blood vessels or under the capsule, contained uniformly sized fat droplets in varying number and concentration. Occasional cells were laden with lipoid. These fat droplets, which were readily demonstrated with sudan IV (Fig. 9) stained dark blue for ketosteroid (Fig. 8). The reaction was readily blocked by prior extraction with lipoid solvents.

Experimental adrenal cortical carcinoma

Tumors of the adrenal cortex have been induced in mice by gonadectomy at birth (Woolley, Fekete and Little, 1943). Their malignant character has been established by transplantation (Woolley and Little, 1946), and their ability to produce androgenic and estrogenic substances has been shown by the development of the accessory sex organs in castrated animals (Woolley and Little, 1945 a, b and c).

A portion of a first generation transplant of such a tumor from a C_3H mouse was fixed in formalin. The ketosteroid reaction in sections of this tumor was very strong. Although there was variation in the amount of ketosteroid-containing lipoid in these tumor cells, nearly every cell contained some positive droplets, and most of the cells were heavily laden with them (Fig. 5). As in the case of normal adrenal gland and tumors in man, ketosteroid and fat was not demonstrated in cell nuclei (Fig. 5). The reactions, used to indicate the ketonic character of the carbonyl group, gave the same results as were obtained with normal adrenal cortex and with the other tumors reported above.

DISCUSSION

Doubt has been raised as to the validity of interpreting the histochemical demonstration of carbonyl groups by hydrazines as indicative of the presence of ketosteroids (Bergner and Deane, 1948). The opinion of those who believe that this reaction may be due to the aldehydic groups of plasmal rather than to ketosteroid is reflected by a recent reviewer, who states, "Histochemists are not yet in a position to identify or localize the steroids of the adrenal cortex. However, Dempsey (1948) and Deane, Shaw and Greep (1948) continue to describe the 'ketosteroids' of the adrenal cortex, despite the overwhelming amount of evidence that the stains which they employ reveal neither active nor inactive ketosteroids in the gland" (Sayers, 1950). The published evidence to which the reviewer referred in order to support this criticism, however, does not warrant so conclusive

a statement. The mere demonstration of carbonyl groups is, of course, not proof of the presence of ketosteroid, even when they are found in substances readily soluble in lipoid. However, since there is evidence (a) that the carbonyl groups so demonstrated are ketonic rather than aldehydic (Seligman and Ashbel, 1951), (b) that they are readily extracted by lipoid solvents from tissues known to contain ketosteroids, such as testicle, corpus luteum, placenta, and adrenal cortex (Ashbel and Seligman, 1949), and (c) that changes in the reaction vary with the functional state of the cells involved (Bergner and Deane, 1948; Deane and Greep, 1946 and 1947; Deane, Shaw and Greep, 1948; Deane, 1951; Deane and Barker, in press; Dempsey, 1948; Greep and Deane, 1947; Jones, 1950; Van Dorp and Deane, 1950; Wislocki, 1949); it is apparent that the reaction does not demonstrate plasmal, but does demonstrate ketosteroid.

Additional support for this conclusion is the following: (a) androgenic steroids have been demonstrated in the lipoid droplets of interstitial cells of the testis by ultraviolet absorption spectromicroscopy (Firminger, 1950). (b) A positive reaction for what we interpret to be ketosteroid was obtained in functioning tumors of the adrenal cortex and in tumors of the ovary, placenta, and testicle (unpublished), while tumors of all other tissues excepting hemangioblastomas of the cerebellum (Seligman and Ashbel), did not exhibit a similar reaction. (c) The demonstration of carbonyl groups not only of the normal adrenal cortex but also in the intracellular lipoid droplets of adrenal tumors fixed in formalin for as long as eleven years, favor the interpretation that they belong to the ketosteroids rather than to the relatively unstable aldehydic carbonyl groups of plasmal. (d) With the exception of the inner elastic membrane of blood vessels (not lipoid soluble), carbonyl groups were not demonstrable in any fresh normal tissue, but were demonstrated, after exposure to formalin for as little as one hour, in the lipoid of various normal tissues known to contain ketosteroid. Exposure of these fresh tissues to agents, which are known to liberate plasmal from plasmalogen, did not produce significant staining for carbonyl groups. No opportunity was afforded for testing the unfixed tissue of four of the tumors reported above, but the tissue of the fifth case and data on tumors associated with Cushing's syndrome, to be reported later, indicate that fixation in formalin is required to unmask the carbonyl groups of ketosteroids in these tumors. The manner in which ketonic carbonyl groups are bound in fresh tissue or the mechanism by which formalin unmasks them is not known. An investigation of this phenomenon is in progress.

Considerable variation in the amount of lipoid and carbonyl-containing lipoid was observed in the different tumors and in various parts of the tumors. In some cells the lipoid was distributed in large droplets (cases 1 and 2), in others in extremely fine droplets (cases 3 and 4). These differ-

ences were sometimes seen within the area of a single field and did not correlate with the malignancy of the tumor. Large areas of many of these tumors, particularly the undifferentiated ones, did not contain cells with demonstrable ketosteroid. Necrotic regions of the tumors were in general free of cells which stained for ketosteroid or lipoid.

It is not possible to distinguish between the various ketosteroids by this method. Since the tumors studied came from patients showing precocious development or virilization or both, the reaction presumably demonstrated at least the androgenic ketosteroids if not the ketonic estrogens or the corticosterones as well. Non-ketonic steroids such as estradiol or steroids with only an 11-keto group are not demonstrable by this method (Ashbel and Seligman, 1949). Since all known ketosteroids have at least a 3-, 17-, or 20-keto group in addition to other groups, we must assume that any of them could have been responsible, in part, for the ketosteroid reaction demonstrated in these functioning tumors. The abnormal amount of 17-hydroxy-corticosteroids in the urine of the first patient suggests that 17-hydroxy-corticosteroids as well as androgens may have contributed to the positive reaction of this tumor.

SUMMARY

Ketosteroids were demonstrated histochemically in tumor cells of five virilizing tumors of the adrenal cortex in man and in an experimentally induced adrenal cortical carcinoma. The case histories are briefly presented and the histochemical results illustrated and discussed.

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OBSERVATIONS ON THE INTERRELATIONSHIP BETWEEN INSULIN, THE ADRENAL CORTEX AND NON-SPECIFIC STRESS (COLD) IN ADIPOSE TISSUE GLYCOGEN SYNTHESIS IN THE RAT

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IN A previous study (Engel and Scott, 1951) it was found that ACTH, cortisone or adrenal extract had no measurable effect on the glycogen concentration of the dorsal interscapular body fat of the rat fasted 48 hours and then fed glucose. On the other hand, administration of insulin with the glucose to rats pretreated with ACTH or cortisone resulted in striking increases in adipose tissue glycogen concentration. This increment was significantly greater than that occurring after treatment with the same dose of insulin and glucose but no adrenal hormone. Insulin either by itself or in conjunction with glucose had been shown earlier to promote glycogen accumulation in adipose tissue (Wertheimer and Shapiro, 1948; Fawcett, 1948; Engel and Scott, 1950). Although circumstantial evidence was presented by Wertheimer and Shapiro (1948) to suggest that the level of glycogen in adipose tissue may reflect the rate of conversion of carbohydrate to fat in this tissue, Engel and Scott (1951) have raised doubts as to whether this interpretation is applicable to the enhanced glycogen accumulation in the ACTH or cortisone treated rats given insulin and glucose.

The ability of insulin to promote glycogen accumulation varies directly with the logarithm of the dose, other factors being kept constant (Engel and Scott, 1950). ACTH at two dose levels and cortisone pretreatment of normal rats given equal doses of insulin and of glucose resulted in approximately the same increase in adipose tissue glycogen levels over glucose-insulin controls regardless of the dose of adrenal hormone (Engel and Scott, 1951). However, since the factor of endogenous pituitary and adrenal activity was a variable in this study, it was considered desirable to determine more quantitatively the comparative roles of insulin and of adrenal hormone in modifying adiopose tissue glycogen concentrations by using adrenalectomized rats treated with varying amounts of cortisone and insulin. At the same time the role of non-specific stress in adipose tissue

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glycogen variations was investigated in intact and cortisone-maintained adrenalectomized rats.

MATERIALS AND METHODS

The analytical methods and hormone preparations used were the same as in previous reports (Engel and Scott, 1950, 1951). Male rats weighing from 140–170 grams at the beginning of the experiment were used throughout. Except where otherwise indicated, the animals were kept in an air-conditioned room at 27–28°C. during the experimental period. This was not possible in the experiments described in the previous reports. Rats were adrenalectomized through the lumbar approach under light pentobarbital anesthesia 2–3 days before being fasted for the experimental procedures. All the adrenalectomized animals received 0.5 mg. desoxycorticosterone subcutaneously daily, and, until the fast was begun, Purina dog chow and 5% glucose and saline in their drinking fountains. At the beginning of the fast, saline was substituted for the glucose in saline. Adrenalectomized rats were fasted 24 hours and intact rats 48 hours before being given 1 ml. of 50% glucose per 100 grams body weight 3 and 6 hours before sacrifice. The dosage schedule for insulin and cortisone acetate will be described below.

TABLE 1. EFFECTS OF INCREASING DOSES OF INSULIN IN 24 HOUR FASTED, CORTISONE-MAINTAINED ADRENALECTOMIZED, GLUCOSE FED RATS

Group	No. of rats	Insulin units/100 gm.	Adipose Tissue glycogen gms./100 gm. fat	p*	Liver glycogen gms./100 gm. liver	p*	Blood sugar mg./100 ml.	p*
A	15	0.5	0.298±0.052		3.85±0.353		120±1.6	
B	16	1.0	0.666±0.098	<.01	2.74±0.210	<.01	117±3.6	n.s.
C	11	2.0	0.985±0.171	n.s. <.01	3.07±0.261	n.s.	90±5.6	<.01

All rats received a total of 17.5 mg. cortisone acetate subcutaneously, 2.5 mg. 72 hours, 5 mg. 48 hours and 10 mg. 24 hours before sacrifice.
All rats received 1.0 ml. 50% glucose/100 gms. p.o. 3 and 6 hours before sacrifice.
* Compared to group A.

RESULTS

Table 1 illustrates the effects of increasing doses of insulin on the adipose tissue and liver glycogen and blood sugar levels of adrenalectomized rats receiving cortisone acetate. These animals were injected subcutaneously with 2.5 mg. of cortisone acetate as a saline suspension on the day of adrenalectomy, 5 mg. on the following day and 10 mg. in 2 divided doses on the third day, the animals being fasted this day. After 24 hours of fasting all rats received 1.0 ml. of 50 per cent glucose/100 grams body weight by stomach tube. At the same time 0.5 unit insulin/100 grams was injected subcutaneously into the animals of group A, 1.0 unit insulin/100 grams into group B and 2.0 units insulin/100 grams into group C. Three hours later the same dose of glucose was administered to all rats and 6 hours after insulin and the first dose of glucose, liver, fat and blood samples were taken under light pentobarbital anesthesia. Examination of the data in Table 1 reveals that the concentration of glycogen in adipose tissue increased with the dose of insulin, although the difference between the 1.0 and 2.0 units

dose levels was not significant. However, previous experience (Engel and Scott, 1951) suggests that if substantially larger groups of animals were used this difference would become significant. When adipose tissue glycogen concentrations are plotted against the dose of insulin on a logarithmic scale a straight line relationship was obtained (Fig. 1) confirming earlier results in intact animals receiving increasing doses of insulin (Engel and Scott, 1950). The concentration of liver glycogen was lower at the 1.0 unit of

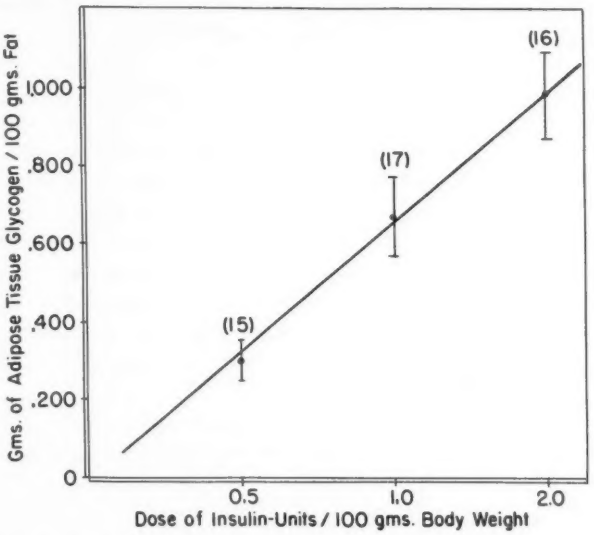

FIG. 1. The effects of increasing doses of insulin on the concentration of glycogen in the dorsal brown fat of cortisone treated adrenalectomized rats given glucose by stomach tube. The insulin doses are plotted on a logarithmic scale.

insulin dose, but not at the 2.0 unit dose, while the blood sugar was depressed only with the largest dose of insulin. These results indicate that when the adrenal factor is kept constant, the concentration of adipose tissue glycogen varies with the dose of insulin. Because of the shorter fast and somewhat different insulin dosage these results cannot be compared quantitatively with the earlier ones from intact rats given cortisone, glucose and insulin.

When the effects of pre-treatment with three different doses of cortisone acetate are compared in animals receiving identical doses of insulin and glucose it is seen that there are no significant differences in the levels of adipose tissue glycogen (Table 2). However, the liver glycogen and blood sugar concentrations are augmented with increasing doses of cortisone, demonstrating that these doses of cortisone exerted effects elsewhere on

TABLE 2. EFFECTS OF INCREASING DOSES OF CORTISONE ACETATE IN 24 HOUR FASTED ADRENALECTOMIZED RATS GIVEN GLUCOSE AND INSULIN

Group	No. of rats	Cortisone mg.*	Adipose tissue glycogen gms./100 gm. fat	p†	Liver glycogen gms./100 gm. liver	p†	Blood sugar mg./100 ml.	p†
D	15	14.0	0.863±0.070		2.20±0.168		107±7.8	
B	17	17.5	0.666±0.098	n.s.	2.74±0.210	<.05	118±3.6	n.s.
			n.s.		p<.01		p<.01	
E	16	27.5	0.645±0.112	n.s.	4.83±0.319	<.01	139±2.6	<.01

All rats received 1.0 unit insulin/100 gms. subcutaneously and 1 ml. 50% glucose/100 gms. p.o. 6 hours before sacrifice and glucose again 3 hours before sacrifice.
* Total dose over 72 hours.
† Compared to group D.

carbohydrate metabolism. In this experiment group D received a total of 14.0 mg. of cortisone acetate intramuscularly over 72 hours, 11.0 mg. being administered during the 48 hours before insulin and glucose treatment, group B was treated as described in Table 1, and group E received a total of 27.5 mg., of which 22.5 mg. were given during the last 48 hours. It would appear from these as well as previous data in intact animals that while cortisone and ACTH potentiate the effect of insulin on adipose tissue glycogen accumulation, this effect is independent of dosage of adrenal hormone, presumably beyond a certain minimal level greater than the endogenous secretion in animals under the experimental conditions previously described (Engel and Scott, 1951).

This being the case, it was of interest to determine whether the presumed stimulation of the pituitary-adrenal system by the stress of exposure to 4°C. during a 48 hour fast would influence the effect of insulin and glucose on adipose tissue glycogen accumulation. The results are seen in Table 3. Four groups of normal rats were fasted for 48 hours, 2 at 4°C. and 2 at 27°C. All animals received 1 ml. of 50% glucose/100 grams by stomach tube 3–6 hours before sacrifice and groups 2 and 3 received in addition 2 units of insulin/100 grams intraperitoneally simultaneously with the first dose of glucose. In each group insulin induced a significant increase in adipose tissue glycogen compared to its control at the same temperature, but in the animals kept at 4°C. and given insulin, the adipose tissue glyco-

TABLE 3. EFFECTS OF COLD STRESS IN 48 HOUR FASTED RATS TREATED WITH INSULIN AND GLUCOSE

Group	Adipose tissue glycogen gms./100 gm. fat		Liver glycogen gms./100 gm. liver		Blood sugar mg./100 ml.	
Glucose—27°C	0.397±0.036 (25)		3.85±0.119 (26)		141±2.7 (26)	
	p<.02		p<.01		p<.01	
Glucose+Insulin 27°C	0.648±0.110 (13)	p<.01	1.93±0.145 (14)	n.s.	72±7.9 (28)	p<.01
	p<.05		p<.01		p<.01	
Glucose—4°C	0.380±0.057 (9)		3.33±0.300 (9)		147±9.3 (9)	
	p<.01		p<.01		p<.01	
Glucose+Insulin 4°C	1.755±0.183 (12)		1.71±0.308 (12)		47±5.4 (23)	

All rats fasted 48 hours and then given 1.0 ml. 50% glucose/100 gm. by stomach tube 3 and 6 hours before sacrifice. Insulin treated rats received 2 units of insulin/100 gm. intraperitoneally with first dose of glucose.

gen concentration was strikingly higher than in all other groups including those receiving insulin at 27°C. The glycogen concentration in the fat of the group exposed to the cold and insulin was also definitely higher than any previous levels seen in animals treated with adrenal hormone and insulin, although in the earlier experiments the dose of insulin was slightly less (Engel and Scott, 1951). Similarly, although the period of fast was longer, the glycogen also was higher than in a comparable group of adrenalectomized animals receiving 17.5 mg. of cortisone acetate in 72 hours and the same dose of insulin and glucose as the above animals (group C, Table 1). Exposure to cold had no influence on the liver glycogen or blood sugar levels of animals given glucose alone. Insulin reduced the liver glycogen

TABLE 4. EFFECTS OF COLD STRESS IN 24 HOUR FASTED CORTISONE-MAINTAINED ADRENALECTOMIZED RATS TREATED WITH INSULIN AND GLUCOSE

Group	No. of rats	Temp. °C	Cortisone mg.	Adipose tissue glycogen gms/100 gm. fat	Liver glycogen gms./100 gm. liver	Blood sugar mg/100 ml.
D	15	27°	14.0	0.836 ± .070	2.20 ± 0.168	107 ± 7.8
				n.s.	$p < .01$	n.s.
F	15	4°	14.0	0.767 ± .088	1.59 ± 0.110	96 ± 6.1
B	17	27°	17.5	0.666 ± 0.098	2.75 ± 0.210	118 ± 3.6
				n.s.	$p < .02$	$p < .01$
G	16	4°	17.5	0.830 ± 0.116	1.97 ± 0.201	88 ± 6.3

All rats received 1 unit insulin/100 gms. 6 hours and 1 ml. of 50% glucose/100 gms. p.o. 3 and 6 hours before sacrifice.

and blood sugar concentrations of both the cold stressed and unstressed rats, but the fall in blood sugar was greater in the former. Since the adrenalectomized animals in group C (Table 1), receiving 17.5 mg. cortisone were better able to sustain their liver glycogen and blood sugar concentrations after 2 units of insulin and 1 gram of glucose per 100 grams than could animals exposed to cold and given the same dose of insulin and glucose, the question may be raised whether some factor other than the adrenal cortex and insulin may not be an influence in promoting the accumulation of glycogen in adipose tissue.

The concept has been receiving increasing attention that the adrenal hormone is not responsible for certain of the metabolic reactions of injury but that it is necessary to facilitate these reactions (Ingle, Ward and Kuizenga, 1947; Engel, 1951). Should this likewise be true with regard to the marked accumulation of glycogen in the adipose tissue of the cold-stressed rat, one would anticipate that it should be possible to demonstrate an influence of cold on the deposition of glycogen in the adipose tissue of cortisone-maintained adrenalectomized rats treated with insulin. This did not prove to be the case in 2 groups of rats receiving 14 and 17.5 mg. of

cortisone acetate respectively over 72 hours and exposed to cold at 4°C. for 24 hours prior to receiving 1 unit of insulin/100 grams and glucose (Table 4). Liver glycogen and blood sugar levels were lower in the animals exposed to cold. Since rather large doses of cortisone were used in this experiment the failure of cold exposure to augment adipose tissue glycogen levels cannot be taken as definitive evidence against adrenal hormone serving only a facilitating role during stress. The high sensitivity of adrenalectomized rats both to exposure to cold and insulin precluded the use of smaller maintenance doses of cortisone.

Because of reports of adrenal hormones and of stress influencing both the size and morphological features of adipose tissue (Schiffer and Wertheimer, 1947; Stoerk and Porter, 1950; Selye and Timiras, 1949) records were kept of the weights of the brown fat carefully dissected from the dorsal interscapular fat pads of the rats in these experiments. Expressed as milligrams of fat per 100 grams of body weight at the time of adrenalectomy, there were no significant differences among the different groups of adrenalectomized animals described above. On the other hand, the weights of the dorsal brown fat of the intact animals exposed to cold and fasted for 48 hours was significantly less than that of the animals fasted at room temperature. The former weighed 114.6 ± 4.9 mg./100 grams initial body weight, the latter 156.4 ± 9.5 mg./100 grams. As expected, the loss of total body weight during the 48 hours fast was greater in the animals exposed to cold.

DISCUSSION

It is apparent that of the various factors under consideration here which influence adipose tissue glycogen, only insulin seems capable of acting independently in altering the net glycogen content of dorsal interscapular adipose tissue. When insulin is administered to the fasted or fed animals, to adrenal hormone treated intact or adrenalectomized rats, or to cold-stressed animals, an increase in adipose tissue glycogen concentration regularly results. On the other hand, neither adrenal hormone in large doses nor cold stress influence the concentration of glycogen in adipose tissue to any measurable degree in the absence of administered insulin. It is tempting to attribute the changes following cold stress to adrenal hormone action secondary to stress, but it is not possible to reach this conclusion on the basis of the experiments described here. However, the data are inadequate for an unequivocal opinion on this point. The levels of the adipose tissue glycogen of the insulin and glucose treated rats stressed by 48 hours exposure to cold are considerably higher than in intact rats receiving 5 mg. of cortisone or 7.5 mg. ACTH over 48 hours or in adrenalectomized rats receiving up to 22.5 mg. of cortisone over 48 hours but

fasted only 24 hours. Furthermore, cortisone and ACTH in both intact and adrenalectomized animals inhibited to varying degrees the fall in liver glycogen and blood sugar following insulin, whereas cold exposure did not. Indeed, animals exposed to the cold and given insulin tended to have lower liver glycogen and blood sugar levels than their corresponding controls at room temperature. This was also true in adrenalectomized animals maintained with large doses of cortisone and exposed to cold. Since increasing doses of cortisone have no effect on adipose tissue glycogen comparable to their effects on liver glycogen and blood sugar in adrenalectomized rats given a constant dose of insulin it is not possible to attribute the increase in adipose tissue glycogen after 48 hour exposure to cold to the effects of a greater secretion of adrenal hormone. At the same time, since exposure to cold does not alter the adipose tissue glycogen concentrations of cortisone-maintained adrenalectomized rats one cannot conclude that the adrenal cortex is serving purely in a facilitating fashion for this metabolic response to stress as is the case in the change in nitrogen metabolism of the stressed animal (Ingle, Ward and Kuizenga, 1947; Engel, 1951). As is now well known, the characteristic change in nitrogen metabolism following stress occurs as well in adrenalectomized animals receiving maintenance doses of adrenal extract as in normal animals. Our experiments described above, however, are not strictly comparable to those on nitrogen metabolism since the doses of cortisone used were very large. One would anticipate that insulin would have an influence on adipose tissue in the presence of this large amount of cortisone and that this might mask any effect which might result from cold exposure. For an animal exposed to cold for 24 hours this response may already be maximal. Further investigation is necessary to determine whether there is a minimal dose of adrenal hormone necessary to sustain the insulin response and whether at this level of adrenal hormone cold stress will accentuate the insulin response. Furthermore, additional information is needed to determine whether the response to cold stress represents a response to non-specific stress in general or whether the cold *per se* is of critical importance. The only intimation of the non-specificity of this reaction is found in the report of Renold, Marble and Fawcett (1950) that a given dose of insulin and glucose induced a greater deposition of glycogen in the adipose tissue of alloxanized rats than in normals.

The present experiments throw no new light on the metabolic pathways of glycogen accumulation in adipose tissue influenced by hormones. However, the recent evidence presented by Welt and Wilhelmi (1950) that ACTH inhibits the conversion of carbohydrate to fat, as measured by the tracer technique with deuterium, offers a basis for speculation. It might be suggested that glycogen accumulation occurs whenever the passage

of glucose into the fat cell exceeds the rate of conversion of glucose (? glucose-6-PO4) to fat in the cell. This is consistent with Wertheimer's evidence that glycogen accumulation precedes fat synthesis and that this glycogen accumulation is generally transitory. Insulin may act by promoting the passage of glucose into the cell as suggested by Levine, Goldstein, Huddleston and Klein (1950) and secondarily thereby, or even directly, also promote the conversion of carbohydrate to fat. In either case it would be necessary to assume that the rate of glycogen synthesis temporarily exceeded the rate of fat synthesis. If adrenal hormone inhibited both conversion of carbohydrate to fat as well as possibly the diffusion of carbohydrate into cell, no net change in adipose tissue glycogen would occur. On the other hand, in the presence of excess adrenal hormone insulin might still effect the increased passage of glucose into the cell, but be unable to break through the inhibition by the adrenal of conversion of carbohydrate to fat. Such an interpretation, while admittedly highly speculative, is subject to experimental investigation by the measurement of the R.Q. of adipose tissues with high glycogen concentrations due to insulin and glucose versus those due to cortisone, insulin and glucose. In the former R.Q.'s over 1.0 might be expected, in the latter a value less than 1.0. This is at present under investigation in this laboratory.

SUMMARY

1. The concentration of glycogen in the interscapular adipose tissue of fasted adrenalectomized rats maintained on a constant dose of cortisone and receiving a fixed amount of glucose by stomach tube increased in direct proportion to the logarithm of the dose of insulin. Blood sugar and liver glycogen concentrations decreased with increasing doses of insulin.

2. The concentration of glycogen in the interscapular adipose tissue of fasted adrenalectomized rats receiving a constant dose of insulin and glucose was not influenced by the amount of cortisone administered. Liver glycogen and blood sugar levels increased with the dose of cortisone.

3. Exposure of normal fasted rats to 4°C. for 48 hours had no influence on the levels of interscapular adipose tissue and liver glycogen or blood sugar after administration of glucose. However, insulin stimulated a striking increase in the interscapular adipose tissue glycogen concentration of the cold-stressed rats compared to control animals at room temperature. Blood sugar levels were lower after insulin in rats exposed to cold.

4. Exposure of cortisone-maintained adrenalectomized rats to 4°C. for 24 hours did not modify the concentration of interscapular adipose tissue glycogen after insulin and glucose compared to similar animals kept at room temperature. Liver glycogen and blood sugar levels were significantly

lower in the cold-stressed cortisone-maintained adrenalectomized rats given insulin and glucose.

5. It was concluded that among those studied insulin is the most important variable in determining the concentration of glycogen in adipose tissue. Possible mechanisms for the alterations in adipose tissue glycogen by insulin and adrenal cortical hormone are considered.

ACKNOWLEDGEMENT

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OBSERVATIONS ON THE RELATION OF RENAL FUNCTION CHANGES TO THE ELECTROLYTE AND GLYCOSURIC EFFECTS OF ACTH IN MAN[1]

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DURING the past few years, the effects of adrenocorticotrophic hormone (ACTH) on carbohydrate, water, and electrolyte metabolism have received considerable attention. The hormone appears not only to decrease carbohydrate tolerance but also to perhaps induce renal glycosuria (Conn, Louis and Wheeler, 1948). In regard to electrolyte metabolism it produces retention of water, sodium, and chloride but promotes loss of potassium in the urine. These alterations in renal function have been thought to be due to the direct effects of the adrenocorticoids on the renal tubules (Conn *et al.*, 1948; Ingle, Li, and Evans, 1946). ACTH and cortisone have been reported to produce changes in renal plasma flow (Ingbar, Relman, Burrows, Kass, Sisson and Burnett, 1950; Berliner) and glomerular filtration rate in man (Ingbar *et al.*, 1950; Berliner; Kendrick, Schoenberger, Dyniewicz, Grimelli and Keeton, 1950), but to have no effect on the maximum ability of the tubules to absorb glucose.

In an attempt to evaluate the role of possible changes in renal function on the electrolyte and glycosuric effects of ACTH, glomerular filtration rate, effective renal plasma flow, and maximum tubular reabsorptive capacity for glucose were followed in a small group of subjects treated with the hormone. At the same time daily excretions of sodium, potassium, and chloride were measured.

MATERIAL AND METHODS

All subjects were studied while on the wards receiving either a regular diet or one providing 250 gms. carbohydrate daily. Patients 2, 4, 5, 6 and 7 had mild rheumatoid arthritis, while patient 3 had rheumatoid arthritis and a bone marrow positive for L. E. cells. All these were afebrile throughout the period of study. Patient 1 was being treated with multiple antibiotics for a resistant subacute bacterial endocarditis due to S. faecalis implantation. The ages of all fell between 23 and 44 years. None had clinical evidence of

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intrinsic renal disease, hypertension, or congestive heart failure during the time of study. Patient 1, however, had experienced an episode of congestive heart failure approximately one year before the study.

The effects of ACTH on the pyrogen reaction, with special reference to the renal hemodynamic changes, were observed in one patient. 0.05 ml. of typhoid-paratyphoid vaccine (N. Y. Board of Health) was administered intravenously to induce the control reaction, and 0.2 ml. for the study after 11 days of therapy.

Daily weights and 24 hour urine collections were started prior to therapy and continued throughout. Aliquots of daily urine specimens and frequent blood samples were analyzed for electrolytes and qualitatively for glucose. Whenever the qualitative test for glucose was positive, a quantitative analysis was made.

Renal function studies were performed according to standard techniques (Goldring and Chasis, 1944) in the morning in the post-absorptive state. Inulin clearance was utilized as a measure of glomerular filtration rate (GFR), p-aminohippurate (PAH) clearance at low plasma PAH levels as a measure of effective renal plasma flow (RPF), while the maximum capacity of the tubules to reabsorb glucose (Tm_G) was measured at high plasma glucose levels. Urine was collected through a multi-eyed catheter and each period was terminated with a bladder rinse with distilled water. Arterial blood samples were obtained in oiled syringes at the midpoints of each period. All results represent the average of three or more consecutive periods.

Serum was utilized for sodium measurements. Dried heparin was used as an anticoagulant in obtaining plasma for the other determinations. Samples for glucose and phosphate analysis were placed in tubes also containing small amounts of fluoride to prevent glycolysis.

An internal standard flame photometer was used for the analysis of sodium and potassium while chloride was determined by a modification of the Vollhard method (Jeffrey, 1927). A modification (Shannon, Farber and Troast (1941)) of the Folin method was used for glucose determinations. Inulin was determined by a modification (Harrison, 1942) of the diphenylamine method (Alving, Rubin, and Miller, 1939) and PAH by the sulfonamide reaction (Bratton and Marshall, 1939).

RESULTS

A summary of the changes in glomerular filtration rates (GFR), renal plams flow (RPF), filtration fraction (FF), maximum tubular reabsorptive capacity for glucose Tm_G), and the GFR/Tm_G ratio before, during and following ACTH is presented in Table 1. These functions have been corrected to 1.73 sq. meters surface area. The initial control weights were utilized for this purpose. The duration of ACTH therapy and the dosages given in the various intervals are indicated.

ACTH induced significant alterations in renal hemodynamics. In six of the seven patients filtration rate increased during hormone therapy. The filtration rate in subject 2 rose from 142 to 223 ml./min. after fourteen days on ACTH, in subject 5 from 96 to 195 ml./min. after eight days, and in subject 6 from 132 to 246 ml./min. at the end of fourteen days on the homone. Increases of lesser magnitude occurred in patients 1, 4, and 7. Following cessation of treatment GFR fell toward or below the control

TABLE 1. EFFECT OF ACTH ON GLOMERULAR FILTRATION RATE, RENAL PLASMA FLOW, AND Tm GLUCOSE IN PATIENTS WITH NORMAL KIDNEYS

Date	Patient number	Surface area	ACTH		Glomerular filtration rate (GFR)	Renal plasma flow (RPF)	Filtration fraction	Tm Glucose (Tm_G)	GFR/Tm_G
			Duration in days	Daily dosage					
		(sq. m.)		(mg.)	(ml./min.)	(ml./min.)	(per cent)	(mg./min.)	
1/18	1	1.63	control		114	483	23.5		
1/25			5	75	119	353	33.7		
2/3			5	75					
			9	100	129	407	31.7		
1/4	2	1.68	control		142	845	16.8		
1/12			5	100	200	771	25.9		
1/19			10	100					
			2	150	223	823	27.1		
2/2			13	no drug	133	885	15.1		
3/31	3	2.00	control		169	553	30.1		
4/25			9	100					
			7	150					
			5	200	125	472	26.5		
3/23	4	1.68	control		107	511	21.0	332	0.323
4/6			11	200	127	389	32.7	333	0.381
4/13			4	no drug	80	350	22.9	293	0.273
2/17	5	1.66	control		96	496	19.4	Tm not reached	
3/3			8	200	195	852	22.9	481	0.406
3/16			13	no drug	113	616	18.3	369	0.307
9/21	6	1.52	control		132			483	0.273
9/30			8	100	142			389	0.365
			9	100					
			5	200	246			518	0.475
9/16	7	1.77	control		128			403	0.317
9/28			6	100	127			387	0.328
			8	100					
			2	150					
			2	200	141			448	0.315

values. In patient 3 with lupus, GFR, which was unusually high in the control period, decreased after twenty-one days of therapy.

In contrast, renal plasma flow fell in four of the five patients in whom it was measured. Two of this group, 1 and 4, demonstrated decreases of more than 100 ml./min., while the decreases in patients 2 and 3 were smaller. RPF manifested a considerable increase in patient 5 after eight days of therapy.

As a consequence of the above changes in GFR and RPF, the filtration fraction increased considerably in three of the five patients in whom it was measured. Only a slight increase occurred in patient 5, who exhibited the rise in renal plasma flow on therapy while a fall in filtration fraction was noted in patient 3 whose GFR decreased 44 ml./min. after three weeks of treatment.

No consistent changes in Tm_G were noted as a result of ACTH. In patient 4 Tm_G did not change while on therapy, but a slight decrease followed termination of ACTH administration. Patients 6 and 7 were observed to have Tm_Gs during ACTH both lower and higher than the pre-treatment values, but the significance of these changes were doubtful. In the other patient Tm_G measured thirteen days following termination of therapy was considerably lower than during treatment. The relationship of filtra-

tion rate to maximal tubular reabsorptive capacity for glucose in this group was higher during therapy than before or after. This appeared to to be due to the marked effect of the hormone on GFR rather than to the slight and inconsistent variations in Tm_G.

The effects of ACTH on daily sodium excretion and GFR in 6 patients are illustrated in Figs. 1 and 2. Values for filtration rate are recorded in the boxes with arrows indicating the day these studies were performed.

At the bottom of Figure 1, patient 4 represents an example of the typical response of daily sodium excretion to ACTH therapy. In this case sodium excretion fell promptly to 1 m.eq./24 hrs. by the second day of treatment, followed by a gradual increase to 39 m.eq./24 hrs. by the eighth day. By the 11th day, GFR had increased from a control value 107 to 127 ml./min. Then, with reduction in dosage prior to withdrawal, sodium excretion increased rapidly and reached a peak of 325 m.eq./24 hrs. the first day

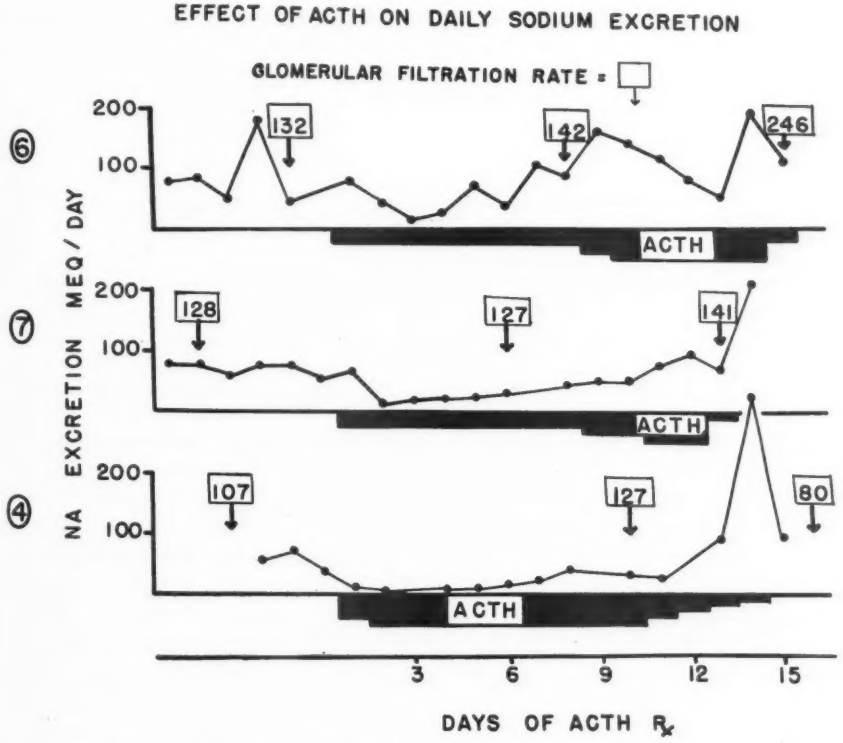

FIG. 1. Effect of ACTH on daily sodium excretion and filtration rate in 3 subjects.

following cessation of therapy while GFR decreased to 80 ml./minute on the 4th day after ACTH was stopped. Serum sodium changed little during the period of study, averaging 132 meq./L.

Sodium excretion was fairly constant in subject 7 (Figure 1) prior to therapy, averaging 66 meq./24 h. but after one day on ACTH decreased to 12 meq./24 hrs. Sodium excretion then gradually increased to 52 meq./24 hrs. by the ninth day although little change in GFR occurred

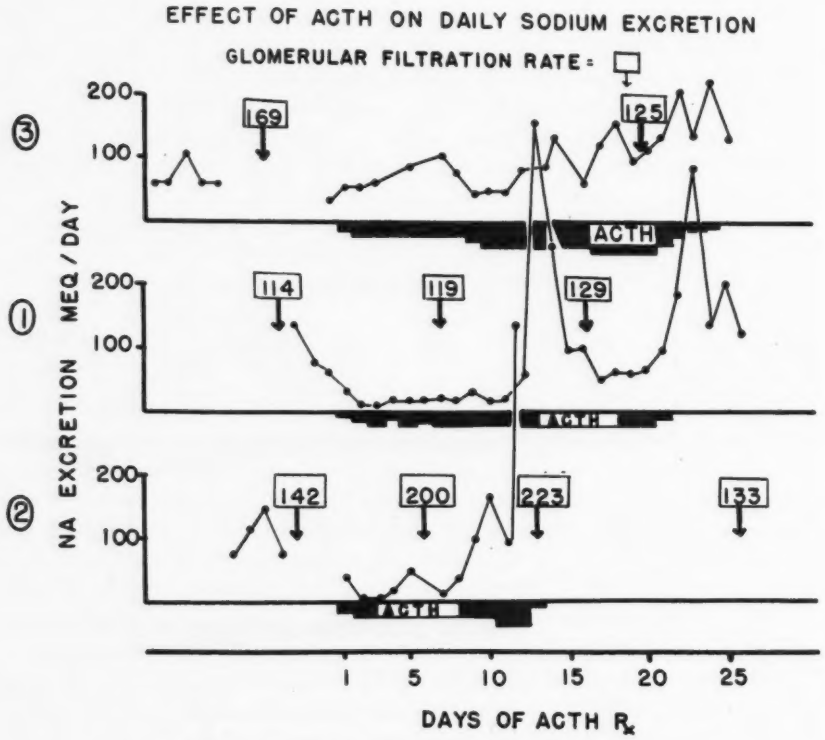

FIG. 2. Effect of ACTH on daily sodium excretion and filtration rate in 3 subjects.

during this period. A further increase in ACTH dosage to 150 to 200 mg. daily for four days did not depress sodium or water excretion, while GFR had increased from 128 to 142 ml./minute. The day following withdrawal of ACTH sodium excretion increased to 209 meq./24 hrs. Serum sodium levels varied between 133 and 138 meq./L. during the control and treatment studies.

In patient 6 (Figure 1), 100 mg. of ACTH daily produced only a transient reduction in the excretion of salt and water. After six days on this dosage sodium excretion increased to levels higher than the average control figures while GFR had increased from 132 to 142 ml./min. When the dosage of ACTH was doubled, a gradual decline in salt output developed which lasted four days. However, on the fifth day of increased dosage it rose to a peak of 186 meq./24 hrs. and GFR increased markedly to 246 ml./minute. In this case a fall in serum sodium from the control value of 140 meq./L to 123 meq./L occurred by the eighth day.

A profound reduction in sodium output early in therapy is well exemplified in subject 2 (Figure 2) whose value fell to 1 meq./24 hrs. on the second day of therapy. Salt excretion then began to increase and GFR increased from 142 to 200 ml./minute after only 4 days of treatment. Late in the course of therapy a marked increase in salt and water excretion was only temporarily affected by increasing the dosage of the hormone, for the following day a peak of 428 meq./24 hrs. was reached. During this diuresis the patient lost 15 pounds and his serum sodium increased slightly. GFR had meanwhile increased further to 223 ml./min. Two weeks after ACTH, GFR had returned to normal.

Observations in patient 1 (Figure 2), the individual with the subacute bacterial endocarditis, were complicated by a fever of approximately 102°F. prior to hormone administration. With the institution of ACTH temperature promptly fell to 99°–100°F. and the day following cessation of treatment it rose to 103°F. Sodium chloride excretion remained low during the first eleven days of treatment. However, between the twelfth and fifteenth days a diuresis with loss of ten pounds occurred. GFR had increased slightly after seventeen days of treatment. Toward the termination of treatment another diuresis commenced which reduced the patient's weight ten more pounds to the control value.

Patient 3 (Figure 2) had had symptoms of rheumatoid arthritis for eight years and prior to treatment L.E. cells were discovered in his sternal marrow at another institution. With the institution of therapy he showed a typical eosinophil response and a fall in his erythrocyte sedimentation rate. However, little or no change in salt or water excretion occurred during therapy, although he gained six pounds during this period. GFR, RPF and FF all fell after twenty-five days of therapy. Serum sodium and plasma chloride did not change significantly.

Potassium excretion among the ACTH treated patients followed no consistent trend, but at one time or another was moderately increased in three of the subjects. Nor did serum sodium or plasma chloride exhibit a consistent pattern. Serum sodium increased in some, decreased in others, and at times underwent no significant changes. Plasma chlorides either

showed no change or increased, while plasma potassium in each case decreased slightly.

The pyrogen reaction is associated with changes in renal hemodynamics (Smith, 1943) inflammatory (Finland, Maxwell, Kass and Ingbar, 1949; Thorn, Forsham, Frawley, Hill, Roche, Staehelin and Wilson, 1950) and pyrogen reactions (Recant, Ott and Fischel, 1950) may be modified by ACTH. Therefore, alterations in renal clearances induced by typhoid vaccine intravenously were observed in one patient before and during ACTH administration. Fever, malaise and muscular discomfort produced by the vaccine were not modified by the hormone in this individual. Glomerular filtration rate increased from 142 to 223 ml./min. during ACTH administration. Equivalent decreases in GFR occurred during the pyrogen reaction induced in the control period and in the ACTH period. Prior to hormone administration the renal plasma flow increased from 822 to 1190 ml./min. during the reaction and after 12 days on ACTH from 802 to 1387 ml./min. In the control period the filtration fraction decreased from 17 per cent to 12.1 per cent during the pyrogen reaction while it decreased from 27 per cent to 12.4 per cent during the pyrogen reaction induced after 12 days of ACTH administration.

DISCUSSION

Administration of ACTH in daily doses varying between 100 and 200 mgm. had definite effects on renal hemodynamics. Glomerular filtration rate increased considerably after a week or more of therapy but showed little change before that time. Decrease in renal plasma flow was observed in several of the patients, while all but one exhibited an increase in filtration fraction. Cessation of hormone administration was followed by reversion of the renal functions to normal within a few days. The increase in filtration fraction together with the rapidity with which the functions changed, either in administration or withdrawal of ACTH, suggest that the observed alterations of glomerular filtration rate and renal plasma flow were on a functional rather than morphological basis. Decrease in renal plasma flow but no change in glomerular filtration rate has been observed after 25–50 mgm. ACTH daily for 7 to 10 days (Berliner), while doses of ACTH up to 400 mg. daily for 3 days increased both GFR and RPF (Ingbar *et al.*, 1950).

That sodium and chloride retention during ACTH administration represents a direct action of increased amounts of corticosteroids on renal tubule cells is most likely. Definite retention of salt and water occurred during hormone administration in five of six patients studied. On the first day of therapy a marked reduction in salt excretion was usually apparent. This occurred in the absence of a decrease in glomerular filtration rate or in

serum sodium. As ACTH administration was continued the rate of sodium and water excertion increased, and by the 10th to 14th day further weight gain generally ceased. A moderate diuresis could occur, even with increased dosage. GFR was frequently increased and may have played some role in this effect. However, equally great diuresis occurred among those patients who exhibited small increases in GFR. Cessation of ACTH was immediately followed by diuresis and reduction in weight below the control values.

Some workers (Conn, Louis, and Wheeler, 1948) have suspected that the glycosuria occurring during ACTH administration was due to a decreased ability of the proximal tubule to completely reabsorb glucose. This remains a definite possibility, although in this study consistent decrease of maximum glucose reabsorption did not occur. Nevertheless, the decreased carbohydrate tolerance and the profound increase in GFR may have presented glucose loads to the tubules in excess of their reabsorptive capacities. In certain patients this might be sufficient to contribute to the glycosuria.

SUMMARY

In a small group of individuals with normal kidneys, renal hemodynamics and electrolyte excretion have been observed during ACTH administration. Glomerular filtration rate increased in most instances. Renal plasma flow usually decreased resulting in an increased filtration fraction. Tm glucose changed little although the GFR/Tm_G ratio usually increased.

Salt and water retention during the administration of ACTH was undoubtedly the result of hormonal action on the renal tubules, although the secondary rise in sodium excretion observed in the patients while still on ACTH therapy was associated with a rise in filtration rate which may have contributed to this effect.

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NOTES AND COMMENTS

THE EFFECT OF ZINC-HYDROCHLORIC ACID HYDROLYSIS ON THE NEUTRAL KETOSTEROIDS IN HUMAN URINE*

In a recent report on ester hydrolysis and extraction of bovine urinary steroids (Arrhenius, 1950) the addition of zinc dust during hydrochloric acid hydrolysis was recommended as a means of reducing extraneous color which is obtained in conjunction with the Zimmermann test (1936–37) for ketosteroids. Others (Smith and Smith, 1937, 1941; Stimmel, 1949) have shown that zinc-hydrochloric acid hydrolysis of the estrogens in human urine converts the phenolic ketonic estrogen, estrone, principally to estradiol. It might be anticipated that the neutral 17-ketosteroids likewise are reduced. Consequently, coincidentally with our studies on the effect of zinc-hydrochloric acid hydrolysis on the estrogens in human urine we have determined the 17-ketosteroid titer (Zimmermann test) of the neutral steroid moiety obtained therefrom.

TABLE 1. NEUTRAL 17-KETOSTEROID TITERS AS DETERMINED BY THE ZIMMERMANN TEST

Exper. No.	Nature of solution for hydrolysis	Nature of Hydrolysis	Titer (in mg. per liter)
1	Pooled human urine	HCl, 15 vol. % 20 min. reflux	8.0
2	Pooled human urine	Zn-HCl 3 hrs. reflux	2.1
3	DDA* (20.0 mg. in 1 liter H_2O	HCl, 15 vol. % 20 min. reflux	17.5
4	DDA* (20.0 mg. in 1 liter H_2O	Zn-HCl 30 min. reflux	0.0

* Dehydroisoandrosterone Acetate

Our results, a few of which are summarized in Table 1, show that the addition of zinc dust during hydrochloric acid hydrolysis of the steroid conjugates in human urine gave only 25 per cent of the 17-ketosteroid titer (Zimmermann test), obtained in the absence of zinc dust. The neutral steroid fractions in Experiments 1 and 2 were treated with Girard's reagent T before application of the Zimmermann test. Hence the difference in 17-ketosteroid titers cannot be attributed to non-specific chromogens. Furthermore, hydrolysis of pure dehydroisoandrosterone acetate (DAA) in aqueous solution by zinc-hydrochloric acid hydrolysis yielded no detectible amount of 17-ketosteroid (Zimmermann test). That these results were due in part to reduction of the 17-keto group is suggested by the following chromatographic and spectrophotometric data.

Chromatography of the neutral steroid residue from zinc-hydrochloric acid hydrolysis of an aqueous solution of dehydroisoandrosterone acetate on a column of activated

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alumina yielded an eluate similar in adsorptive characteristics to that of Δ-5 androstene-3,17-diols. Also comparison of the sulfuric acid color spectrum of this eluate showed absorption maxima (410 mu, 490 mu) similar to those obtained with authentic specimens of Δ-5 androstene-3(β), 17(α), diol and Δ-5 androstene-3(β), 17(β), diol.

Addition of zinc dust during hydrochloric acid hydrolysis of the steroid conjugates in human urine therefore cannot be expected to yield reliable information regarding neutral 17-ketosteroid excretion as determined by the Zimmermann test. One might assume that the same conditions prevail in urine from other than human sources but we have not had opportunity to investigate this point.

We are indebted to Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J. for the Δ-androstene-3(β), 17(α), diol and Δ-5 androstene-3(β), 17(β), diol used in this study.

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COMPARISON OF THE FORMALDEHYDOGENIC LIPID OF URINE BEFORE AND AFTER GLUCURONIDASE HYDROLYSIS

Kinsella, Doisy and Glick (1950) have suggested the use of a bacterial beta-glucuronidase for the hydrolysis of urinary steroids of the cortico-steroid type. Utilizing a modification of the technique of Heard, Sobel and Venning (1946) they were able to note an increase in the neutral reducing lipid of 3–5 times that extracted from unhydrolyzed urine.

We have attempted to adapt this enzymatic hydrolysis to the measurement of urinary formaldehydogenic steroids (cortin) (Daughaday, Jaffe and Williams, 1948). Maximum liberation of formaldehydogenic material was obtained when 50 cc. of urine was incubated with 3200 units (roughly equivalent to 800 Fishman units) of the beta-glucuronidase preparation prepared according to the directions of Buehler, Katzman and Doisy (1949) at 37° for 18 hours. The urine was then extracted and the formaldehyde liberated after periodic acid oxidation was measured using chromotropic acid.

Parallel determinations with and without enzymatic hydrolysis have been performed on 34 urine samples from normal individuals, patients without adrenal disease and patients with adrenal cortical disease. The results are presented in Figure 1. There is no evident correlation between the formaldehydogenic steroids of the unhydrolyzed and enzyme hydrolyzed urine samples.

By the study of urine samples from patients with hypo and hyper function of the adrenal cortex the significance of enzymatic hydrolysis was evaluated. Assays performed on patients with Addison's disease and Cushing's syndrome are listed in Table 1.

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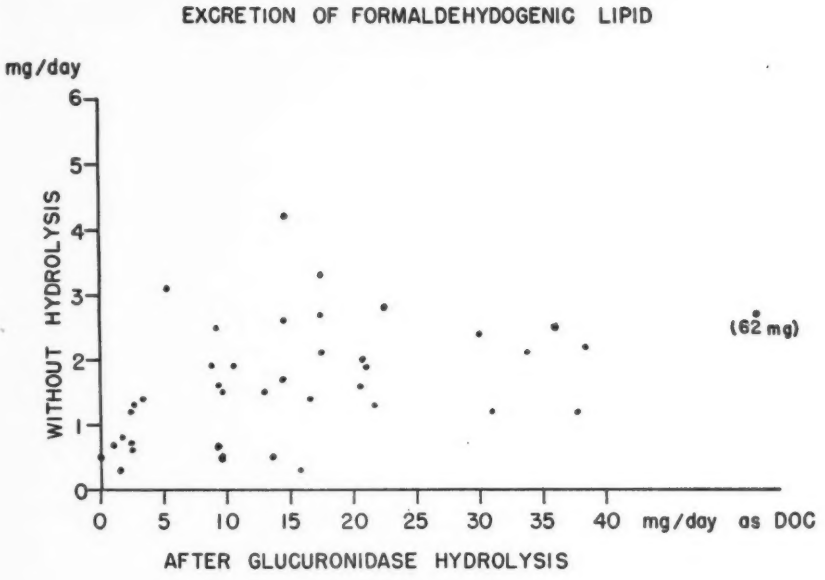

FIG. 1

We have concluded from our studies that the bacterial enzyme preparation liberated considerable formaldehydogenic material which is not correlated with the excretion of formaldehydogenic material in unhydrolyzed urine. That this material need not arise from the adrenal cortex is suggested by the high levels of excretion in two of four individuals with Addison's disease. Identification of these liberated formaldehydogenic compounds is in progress.

TABLE 1

Patient	Sex	Diagnosis	Formaldehydogenic Steroids		Reducing Steroids enzyme treated mg./day	17-Ketosteroids untreated mg./day
			Untreated mg./day	Enzyme treated mg./day		
J.D.	M	Addison's Disease	0.7	29.2 21.4	9.1	6.8 5.3
W.L.	M	Addison's Disease	0.3	1.7		3.1
L.G.	F	Addison's Disease	0.5	0		2.0
B.M.	F	Addison's Disease	0.3	15.9	1.8	1.8
F.B.	F	Cushing's Syndrome	2.4 4.2	29.0 13.7		18.5 22.7

Enzymatic hydrolysis does not increase the clinical significance or specificity of the measurement of urinary formaldehydogenic steroids.

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